Die Biogenese der Haut

Etablierung von mRNA Expressionsprofilen von proliferierenden und differenzierenden Keratinozyten unter Einfluss von Stickstoff Monoxid

und

Untersuchung einer Glucosylceramide Synthase defizienten Maus

Dissertation

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Abbreviations

Abbrviations

Free Extractable Ceramides:

Ceramide AH: contains 6-Hydrxysphingosin (H) and α-Hydroxy-Fatty Acid (A)

Ceramide AP: contains Phytosphingosine (P) and α-Hydroxy-Fatty Acid (A)

Ceramide AS: contains Sphingosine (S) and α-Hydroxy-Fatty Acid (A)

Ceramide EOH: contains 6-Hydrxysphingosin (H) and an ω-Hydroxy-Fatty acid (O) ester bound to linoleic acid

Ceramide EOP: contains Phytosphingosine (P) and an ω-Hydroxy-Fatty acid (O) ester bound to linoleic acid

Ceramide EOS: contains Sphingosine (S) and an ω-Hydroxy-Fatty acid (O) ester bound to linoleic acid

Ceramide NH: contains 6-Hydrxysphingosin (H) and a non-Hydroxylated Fatty Acid (N)

Ceramide NP: contains Phytosphingosine (P) and a non-Hydroxylated Fatty Acid (N)

Ceramide NS: contains Sphingosine (S) and a non-Hydroxylated Fatty Acid (N)

Protein-bound Ceramides:

Ceramide OD: contains Dihydrosphingosine (D) and an ω-Hydrox-Fatty Acid (O)

Ceramide OH: contains 6-Hydrxysphingosin (H) and an ω-Hydrox-Fatty Acid (O)

Ceramide OP: contains Phytosphingosine (P) and an ω-Hydrox-Fatty Acid (O)

Ceramide OS: contains Sphingosine (S) and an ω-Hydrox-Fatty Acid (O)

Abbreviations

BH4: Tetrahydrobiopterin

BSA: Bovine Serum Albumine

BPE: Bovine Pituitary Extract

CBE: Conduritol-B-Epoxid

CERT: CERamide Transport protein

cpm: Counts Per Minute

DMEM: Dulbecco's Minimal Essential Medium

DTT: Dithiotreitol

EDTA: Ethylendiamin tetraacetic acid

EGF: Epidermal Growth Factor

ESI: ElectroSpray Ionisation

FB1: Fumonisin B1

FCS: Fetal Calf Serum

GlcCer: GlucosylCeramide

HEK: Human Epidermal Keratinocyte

Hepes : N-[2-Hydroxylethyl]piperazin-N`-[2-ethansulfonic acid]

HPTLC: High Performance Thin Layer Chromatography

L-NAME: N^G-nitro-L-arginine-methylester

MS/MS: Fragmentation Mass spectrometry

MuLV RT: Murine Leukimia Virus Reverse Transcriptase

NO: Nitric Oxide

NOS: Nitric Oxide Synthase

PBS: Phosphate Buffered Saline

SNAP: S-nitroso-N-acetyl-D,L-penicillamine

SPT: Serine PalmitoylTransferase

SapX: Sphingosine activator protein X (A, B, C, or D)

TEWL: TransEpidermal Water Loss

TLC: Thin Layer Chromatography

TNF-α: Tumor necrosis factor

Tris: Tris-(hydroxymethy)-aminomethan

TUNEL: Terminal desoxyribosyl-transferase mediated dUTP nick end labelling

v/v: Volume to Volume

W/V: Weight to Volume

Content

Content

1. Summary

Summary

The epidermis is a histologicaly highly complex organ. It is composed of cells in different phases of their cell cycle. They are either proliferating (in the lowest layer, the basal layer), or undergoing different phases of a process leading to terminal differentiation. This results in the formation of an outer layer composed of dead keratinocytes enclosed in a scaffold made of a 3 dimensional protein structure to which lipids, predominantly long chain ceramides and long chain fatty acids, are covalently attached. These lipids form the permeability barrier which plays a crucial role in maintaining water homeostasis, an essential feature of the skin of land dwelling organisms.

In order to be able to study the formation of this barrier, we established in our laboratory a submerged culture system which, when accordingly modulated, enables keratinocytes to proliferate (in low calcium medium 0.1 mM) or initiate differentiation (at a high calcium concentration of 1.1 mM). These cells undergo the different stages of terminal differentiation, but do not reach the stage of *stratum corneum*. These cells synthesise most of the ceramides, especially the long chain ceramides necessary for barrier formation. We sought to characterize the different conditions under which keratinocytes were cultivated. These conditions include low calcium medium (0.1 mM), high calcium medium (1.1 mM), high calcium medium with linoleic acid (10 µM), and high calcium medium with vitamin C. We measured the mRNA expression profiles of several enzymes of the ceramide metabolism (serine palmitoyltransferase, acid ceramidase, acid sphingomyelinase, glucosylceramide synthase, and β-glucocerebrosidase), as well as several markers of proliferation (keratin 14) and differentiation (keratin 10), and proteins involved in the formation of the protein matrix of the permeability barrier (profilaggrin).

The calcium shift induced keratinocytes to differentiate, as the mRNA of the proliferation marker keratin 14 declined, while that of keratin 10 increased 75-fold. This calcium shift triggered an increase in mRNA levels of profilaggrin (55-fold), glucosylceramide synthase (40-fold), β-glucocerebrosidase (30-fold), prosaposin (15-fold), acid sphingomyelinase (5-fold), and serine palmitoyltransferase (subunit LC2, 4-fold). However, mRNA levels of acid ceramidase did not change significantly. The addition of linoleic acid increased the amounts of all the markers, while vitamin C did not show any effect.

1. Summary

We studied the effect of nitric oxide on keratinocyte differentiation by the addition of either the NO donor S-nitroso-N-acetyl-D,L-penicillamine (SNAP, 0.2 mM) or the NO synthase inhibitor N^G-nitro-L-arginine-methyl ester (L-NAME, 10 mM), a prodrug.

Nitric oxide added at concentrations lower than 0.25 mM stimulates proliferation of keratinocytes. Accordingly, SNAP had no effect on the morphology of cultured keratinocytes, whereas in cultured human fibroblasts apoptosis was induced. The expression patterns obtained suggest that keratinocytes remain in a basal proliferative state, with a 3-fold increase in keratin 14 expression, a marked decrease in mRNA levels of differentiation markers and of most ceramide metabolizing enzymes to negligible levels. L-NAME induced a transient increase in ceramide formation, followed by apoptosis in keratinocytes but not in fibroblasts. SNAP as well as L-NAME, decreased the mRNA levels of all proteins involved in ceramide metabolism.

Ceramides are important components of the water- and electrolyte barrier in the skin of mammals. These ceramides contain ω-hydroxylated very long chain fatty acids in their lipid moiety which, in addition, are found to be esterified with linoleic acid. Glucosylceramide (GlcCer) is thought to be a carrier of these very special ceramides. To investigate the role of GlcCer in epidermal barrier function a mouse with glucosylceramide synthase deficiency in the epidermis was generated. Four days after birth deletion of GlcCer resulted in pronounced skin desquamation, associated with extreme transepidermal water loss. Severe structural defects were observed as the epidermis of the mutant mice was extremely broadened, showing hyperproliferative keratinocytes, increased apoptosis, detached stratum corneum, and unusual distribution of epidermal keratins. Lamellar bodies of the *stratum granulosum* showed an irregular structure and corneocytes of the *stratum corneum* were tightly clustered. All mice died five days after birth due to the pronounced skin defects. The deletion of the glucosylceramide synthase gene induced multiple alterations in skin leading to a breakdown of barrier function of the skin and death. We have found that keratinocytes have a compensatory mechanism which compensates for lost GlcCers by way of sphingomyelins, possibly long chain sphingomyelins with ω ester fatty acids. But this compensatory pathway seems to be either too slow, or not well adapted to the function of barrier build-up. It is possible that the phosphocholine headgroup of sphingomyelin is not adapted to the task of creating the highly structured assembly system of the permeability barrier.

2. Introduction

2.1. General information

The skin is often known as "the largest organ of the human body". This applies to exterior surface, as it covers the body, *appearing* to have the largest surface area of all the organs. Moreover, it applies to weight, as it weighs more than any single internal organ, accounting for about 15 percent of body weight. For the average adult human, the skin has a surface area of 1.5-2.0 m^2, most of it 2-3 mm thick. The average square inch of skin holds 650 sweat glands, 20 blood vessels, 60,000 melanocytes, and more than a thousand nerve endings.

The skin is made up of multiple layers of epithelial tissues that guard underlying muscles and organs. As an interface with the environment, it plays the most important role in protecting against pathogens. Its other main functions are insulation and temperature regulation, as well as sensation. Skin is considered one of the most important parts of the body. For land dwelling organisms, the skin has also a primordial function of preventing transepidermal water loss. Skin has pigmentation, or melanin, provided by melanocytes, which absorb some of the potentially dangerous ultraviolet radiation in sunlight. It also contains DNA repair enzymes which help to reverse UV damage, and people who carry mutations in the genes coding for these enzymes suffer high rates of skin cancer. One form predominantly produced by UV light, malignant melanoma, is particularly invasive, spreads quickly, and can often be deadly. On some animals the skin is very hard and thick. Reptiles and fish have hard protective scales, and birds have hard feathers, all made of tough β-keratins. In contrast, Amphibian skin is not a strong barrier to passage of chemicals and is often subject to osmosis. A frog sitting in an anaesthetic solution will quickly fall asleep.

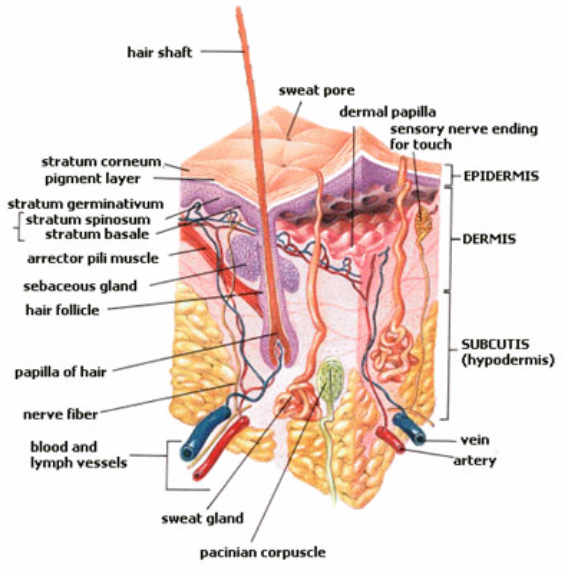

Fig 1. The layers of the skin

2.2. The layered structure of the skin

The skin is composed of three primary layers: the *epidermis*, which provides waterproofing and serves as a barrier to infection; the *dermis*, which serves as a location for the appendages of skin; and the *hypodermis (subcutaneous adipose layer)*, also called the basement membrane. The outermost epidermis consists of stratified squamous epithelium. The epidermis contains no blood vessels, and cells in the deepest layers are nourished by diffusion from blood capillaries extending to the upper layers of the dermis. The main types of cells which make up the epidermis are keratinocytes, with melanocytes (pigmentation, UV protection) and Langerhans cells (antigen detection and presentation) also present. The epidermis can be further subdivided into the following *strata* (beginning with the innermost layer): *basale* (SB), *spinosum* (SS), *granulosum* (SG), *lucidum* (SL), and *corneum* (SC).

The *stratum basale* is the proliferative strata where keratinocytes undergo mitosis. The cells have big nuclei and synthesize keratins 5 and 14 (Byrne et al. 1994) . At a certain point, cells stop proliferating and undergo a differentiation program. The signal for the start of differentiation is the extracellular Ca^{2+} level (Hennings et al. 1980), but other factors can also play a role, such as Vitamins A (Fuchs and Green 1981), C (Savini et al. 2002) and D (Bikle et al. 2001), UV radiation (Haratake et al. 1997; Hendrix et al. 1998), cytokines (Hammerberg et al. 1998), growth factors (Beer et al. 2002), and cell-cell contact (Furukawa et al. 1997). In the *stratum* spinosum, keratinocytes acquire a polygonal shape and lose their capacity to proliferate while becoming metabolically highly active. They have a high number of desmosomes to anchor keratins, predominantly keratins 1 and 10. This allows for a high mechanical stability as the keratin filaments become more and more tightly packed (Kirfel et al. 2003). The cells then transit to the *stratum granulosum* were they become flatter and start losing organelles. They produce keratohyalin granules containing profilaggrin which, when processed to filaggrin at later stages, increase the packing density of keratin filaments (Nemes and Steinert 1999) which are oriented parallel to the cell surface. At this stadium, cells start synthesizing lamellar bodies containing glucosylceramides (called probarrier lipids), phospholipids, and acid hydrolases, which appear to originate from the trans-Golgi network (Elias et al. 1998). The *stratum lucidum* is made of the first few cell layers of the *stratum corneum.* The cells are optically denser, and this layer is more pronounced in the skin covering the interior of the hands and the feet soles. The SC is the outer layer of the epidermis. It is 8-13 μm thick and the keratinocytes it contains have lost all metabolical activity as well as most of their organelles. The cells are flat and called corneocytes. They are

full of keratin filaments which, together with other proteins such as loricrin, Small Proline Rich proteins, involucrin, trichohyalin and several other proteins, create a 3D matrix through disulfide and isodipeptide bonds, the latter catalyzed by Ca^{2+}-dependent transglutaminases 1, 2, 3, and 5 (Candi et al. 2001; Eckert et al. 2005). This matrix is 10 nm thick and replaces the plasma membrane of corneocytes. It is externally covered with a monolayer of covalently bound lipids, and then by a multilayer of other lipids (cholesterol, fatty acids, and ceramides), which are not bound to the protein matrix (Kalinin et al. 2002).

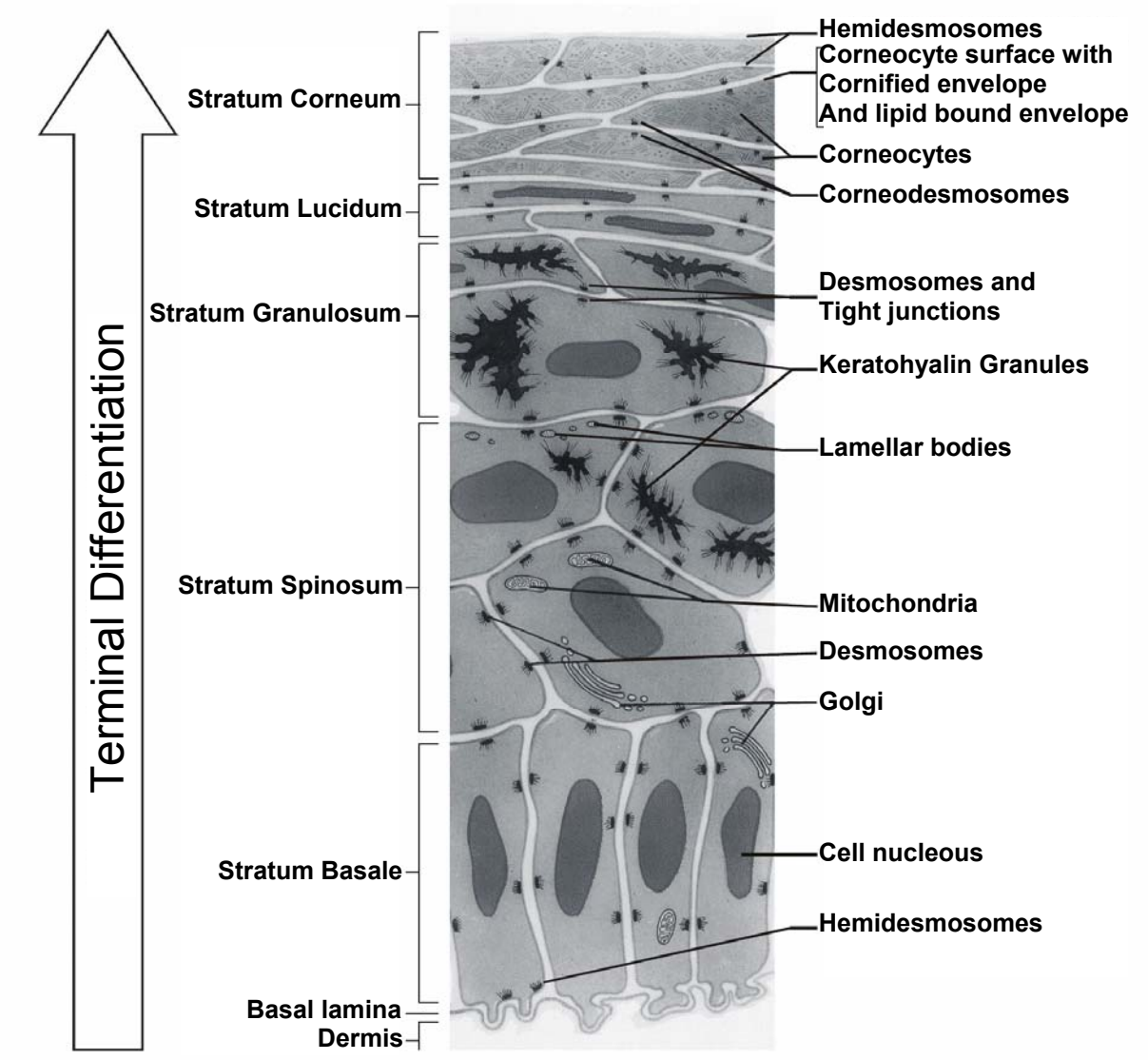

Fig. 2 Structure of the epidermis. (modified after (Montagna et al. 1992). The layer structure of the epidermis shows the differentiation stages of keratinocytes. The whole epidermis is 0.04 mm (eye lid) to 1.2 mm (foot sole) thick. Keratinocytes take about 2 weeks to reach the SG, and further 2 weeks for the SC.

2. Introduction

An important component of the integrity of the epidermis is composed of the tight junctions. These junctions are connections between adjoining cells which allow a very close adhesion of the cells to each other. This permits the filling and sealing off of the intercellular space between the cells (Morita and Miyachi 2003).

The integral membrane proteins occludin and claudin, main constituents of tight junctions, have been detected in murine (Morita et al. 1998; Furuse et al. 2002) and, more recently, in human epidermis (Pummi et al. 2001; Brandner et al. 2002). By restricting paracellular diffusion, tight junctions are essential for the barrier function of simple epithelia (Balda and Matter 1998), but they also contribute to the formation of the epidermal diffusion barrier, as recently demonstrated by Furuse et al. (Furuse et al. 2002). The importance of tight junctions for the permeability barrier of the skin is shown by the Claudin-1 knock out mouse. These mice die 1 day after birth due to an elevated transepidermal water loss (Furuse et al. 2002).

Terminal differentiation of keratinocytes takes place within 30 days (Watt 1988) and is a dynamic steady state, as cells are continuously produced in the SB and corneocytes slough off from the SC (desquamation). It results in the formation of an epidermal permeability barrier, consisting of a densely packed system of corneocytes embedded in a multilamellar extra cellular lipid matrix (Watt 1989; Fuchs 1990).

2.3. Cell culture of keratinocytes

In order to study the metabolical processes taking place during proliferation and differentiation of keratinocytes, it is necessary to establish a cell culture model which is biochemically as close to the epidermis as possible. Two types of cell culture systems have been developed, the submerged model and the 3D culture. The latter is based on the culture of cells at the air-culture medium interface, with supporting tissues such as fibroblasts (Limat et al. 1996; Gibbs et al. 1997). These tissues are able to provide to keratinocytes the necessary supplements for growth and differentiation through diffusion (as the epidermis does not have capillaries), as well as mimic the original state of keratinocytes in the epidermis. The second type of culture, the submerged culture, enables the production of a monolayer, depending on the medium type (Hennings et al. 1980). Previous studies (Döring 1999; Breiden 2003) have shown the effect of different substances on keratinocyte differentiation. These led to the establishment of a submerged culture system leading to differentiated keratinocytes. This

system consists in the culture of keratinocytes in MCDB-153 medium at low calcium concentration (0.1 mM, low calcium medium). This medium enables keratinocytes to proliferate. At confluence, the medium is changed to MCDB-153 with a calcium concentration of 1.1 mM, to which 10 µM of a linoleic acid-BSA complex is added (high calcium medium). This calcium shift induces keratinocytes to differentiate to the different stages of the epidermis described earlier. These culture conditions were characterized previously using lipid analysis (Döring 1999; Breiden 2003). In this study, we reproduced the culture conditions described previously and measured the mRNA expression levels of different proteins and enzymes involved in terminal differentiation and ceramide metabolism.

2.4. The ceramides

2.4.1. Ceramide structure

Ceramides are the major components of the lipid barrier of the *stratum corneum*, which also contain cholesterol and free long chain and very long chain fatty acids (Wertz et al. 1985; Weerheim and Ponec 2001). Ceramides are of critical importance not only for permeability barrier homeostasis, but also for intracellular signalling processes that regulate proliferation and apoptosis (Geilen et al. 2001). To date, 9 different types of ceramides have been described (Stewart and Downing 1999; Ponec et al. 2003). Ceramides are composed of a sphingoid base and a fatty acid. The sphingoid base can be sphingosin (S), phytosphingosin (P), or 6-hydroxysphingosin (H). It has a chain length of 16 and 26 carbon atoms, and, in principle, also sphinganine (D) (Wertz and Downing 1987). The fatty acid can be non-hydroxylated (N; 14-30 carbon atoms); α-hydroxylated (A; 16-28 carbon atoms), or ω-hydroxylated (O; 24-36 carbon atoms). Free ceramides with an ω-hydroxylated fatty acid have a linoleic acid ester in the ω position. The chain may have odd or even carbon atoms (Wertz and Downing 1987), and can be polyunsaturated (Wertz et al. 1989). This forms the basis of the general nomenclature used for ceramides according to Motta (Motta et al. 1993), where the name (Cer(XY)) is derived from the letter for the fatty acid (X: N, H, or O), followed by that of the sphingoid base (Y: S, P, D, or H). An alternative nomenclature uses the R_f-value of the Ceramides separated on TLC plates (Cer 1 to Cer 9), where Cer 1 has the highest R_f-value, and Cer 7 the lowest (Bouwstra et al. 2003). Ceramides 8 and 9 were discovered later (Stewart and Downing 1999; Ponec et al. 2003).

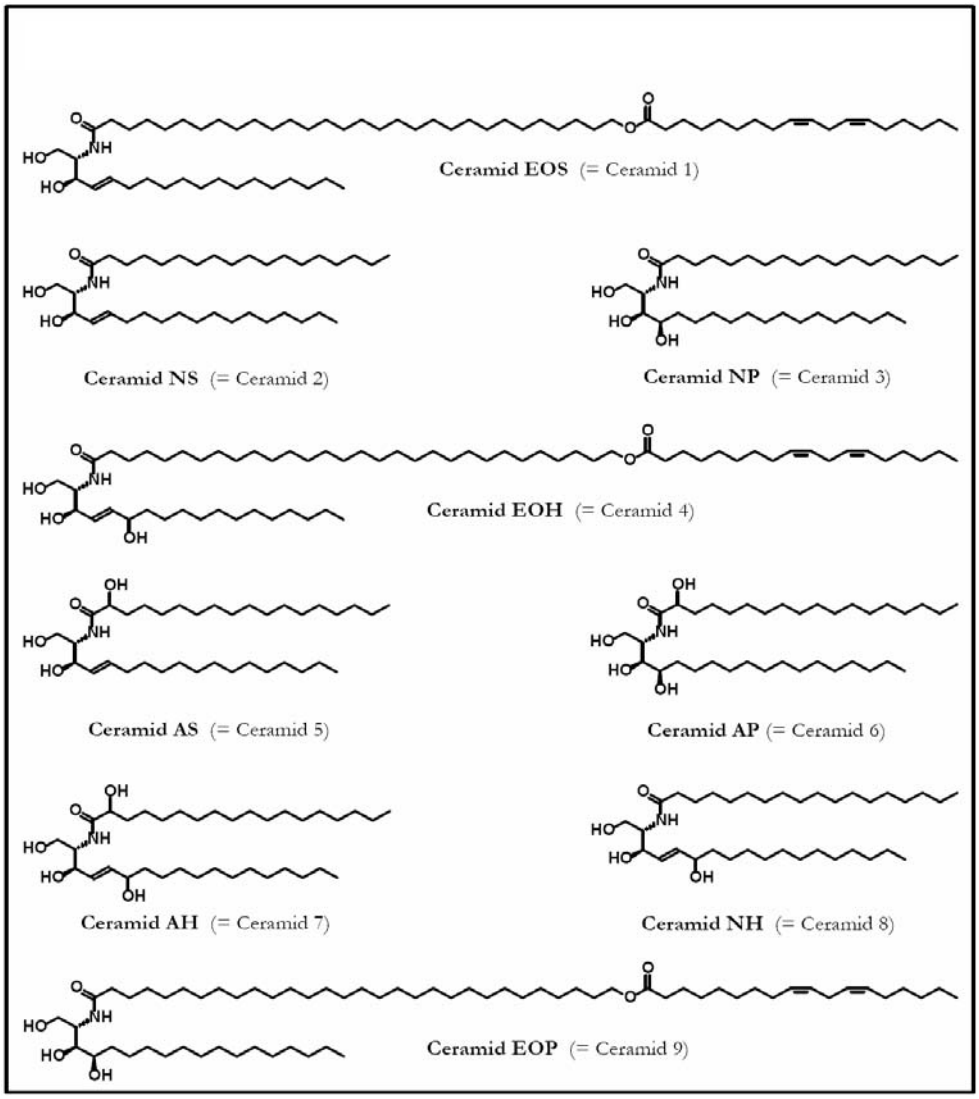

Fig. 3 Ceramide structures in the nomenclatures of (Motta et al. 1993) and (Ponec et al. 2003) (between parenthesis). The first letter describes the fatty acid (N: non-hydroxylated FA; A: α-hydroxylated FA; O: ω-hydroxylated FA; E: Linoleic acid ester) and the second the sphingoid base (S: Sphingosin; P: Phytosphingosin; H: 6-Hydroxysphingosine). A further sphingoid base, sphinganine, is identified by the letter D. In parenthesis is the ceramide nomenclature according to the R_f-value in a TLC.

To these ceramide species we have to add those which are covalently bound in the human epidermis. These are Cer(OS), Cer(OH), Cer(OP), and Cer(OD).

2.4.2. Ceramide biosynthesis

The *de novo* biosynthesis of ceramide takes place at the cytosolic face of the endoplasmic reticulum and necessitates 4 steps. It starts with the condensation of L-serine with palmitoyl or stearoyl-CoA forming 3-ketosphinganine by the action of the serine palmitoyltransferase (SPT). SPT, a pyridoxal 5-phosphate-dependent decarboxylase, is the rate-limiting enzyme of the pathway. 3-Ketophinganine is then reduced to D-erythro-sphinganine (Stoffel et al. 1968). Dihydroceramide synthase (DHS), a sphinganine-N-

acyltransferase, catalyzes the formation of an amide linkage between a long chain (typically saturated) fatty acid and the amino group of sphinganine (Merrill and Wang 1986) and results in the formation of 4,5-dihydroceramide. In keratinocytes, the fatty acid can have a chain length of up to 36 carbon atoms. DHS is the target of a family of mycotoxins (fumonisins) that cause a wide spectrum of disease symptoms (neurotoxicity, hepatotoxicity, and nephrotoxicity, pulmonary edema, cancers of liver, kidney, and esophagus, birth defects, and possibly other disorders). This is an indication of the wide spectrum of action of ceramides and other components of its metabolism. And finally, dihydroceramide desaturase (DHD) and hydroxylase (DHH) introduce the 4-*trans* double bond (to produce acylated sphingosine) and the C-4 hydroxyl of acylated phytosphingosines, respectively (Rother et al. 1992; Michel and van Echten-Deckert 1997; Michel et al. 1997). The desaturase reaction is critical because it converts an essentially innocuous precursor (dihydroceramide) into a highly bioactive product (ceramide and downstream species such as sphingosine and their derivatives).

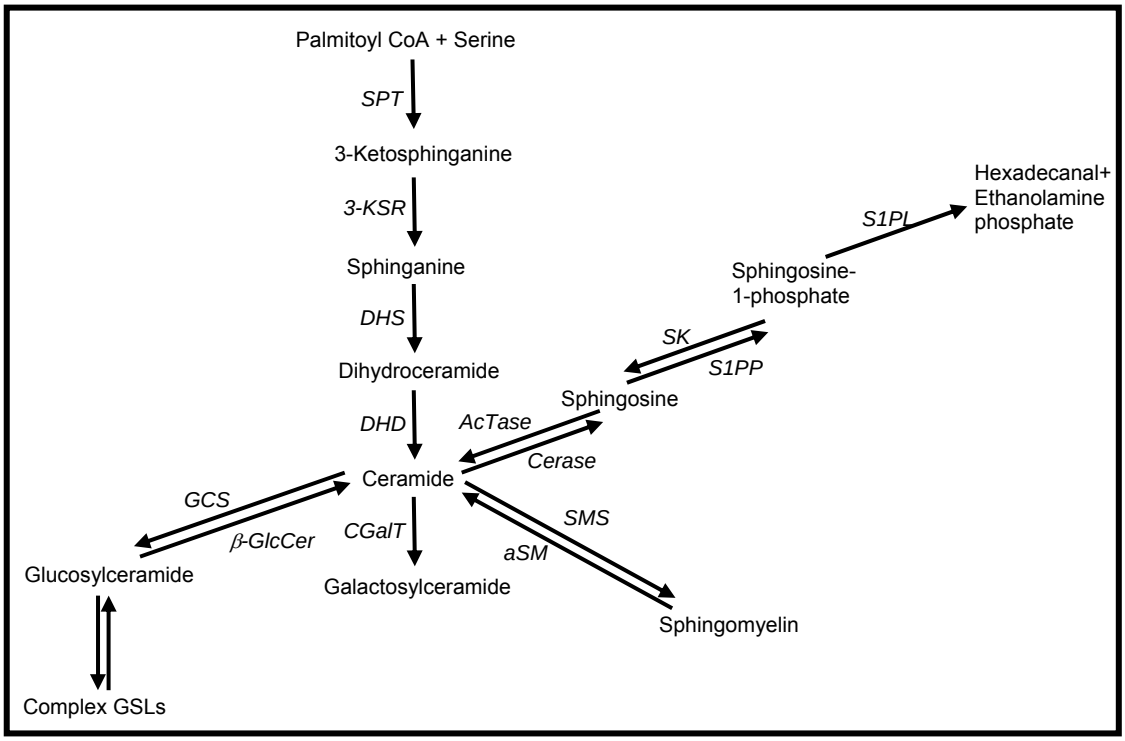

Fig. 4 The biosynthesis of sphingolipids and glycosphingolipids (GSL). Enzymes are shown in italic. SPT: Serine Palmitoyl Transferase; 3-KSR: 3-Ketosphinganine reductase; DHS: Dihydroceramide Synthase; DHD: Dihydroceramide Desaturase; GCS: Glucosylceramide Synthase; GlcCer: β-Glucocerebrosidase; Cerase: ceramidase; AcTase: Acyltransferase; CGalT: Ceramide Galactosyl Transferase; aSM: acid Sphingomyelinase; SMS: SphingoMyeline Synthase; SK: Sphingosine Kinase; S1PP: Sphingosine-1-phospate phosphatase; S1PL: Sphingosine-1-phosphate lyase.

2. Introduction

In order to synthesize both glucosylceramide and sphingomyelin, ceramide must be transported from the ER to the Golgi apparatus (Jeckel et al. 1992; Lannert et al. 1994). One important development in this field in recent years was the identification of a protein, CERT, which mediates the transport of ceramide from the ER to the Golgi apparatus in a non-vesicular manner (Hanada et al. 2003). CERT is a cytoplasmic protein with a phosphatidylinositol-4-monophosphate-binding domain and a putative domain for catalysing lipid transfer. This protein specifically extracts ceramide from phospholipid bilayers and shows very little activity towards other related SLs or glycerolipids. CERT also extracts dihydroceramide but displays a relatively restricted fatty acid specificity, efficiently mediating the transfer of ceramides containing C14–C20 fatty acids but is less efficient towards ceramides containing longer-chain fatty acids (Kumagai et al. 2005). CERT is involved in the delivery of ceramide for SM synthesis (Fukasawa et al. 1999), but not for GlcCer synthesis, which seems to depend on the supply of ceramide through a distinct ATP- or cytosol-independent (or less dependent) pathway (Riezman and van Meer 2004). Thus, at least two mechanisms exist for delivering ceramide from the ER to the Golgi apparatus. On the cytosolic side of the Golgi membrane, a glucose is attached to the ceramide at the C1 position. This is done by the glucosylceramide synthase (GCS). Another possibility is for ceramide to flip directly to the luminal side of the Golgi membrane, where a galactose or phosphocholine residue is attached. The GlcCers flip to the luminal side of the Golgi membrane, where the glycosphingolipid biosynthesis pathway is completed by the subsequent attachment of carbohydrates, sulfate (sulfitides) or neuraminic acid (gangliosides) residues (Lannert et al. 1998). Ceramide can also be formed by sphingomyelinase-catalyzed turnover of sphingomyelin (aSM) (Hassler and Bell 1993) or enzymatic hydrolysis of glucosylceramide (β-Glucocerebrosidase). The importance of GCS is shown by deletion experiments where its disruption was embryonically lethal during gastrulation at embryonic day 6.5 (E6.5) to E7.5 (Yamashita et al. 1999). Furthermore, the brain specific deletion of GCS in mice led to severe neural deficiencies after birth (Jennemann et al. 2005).

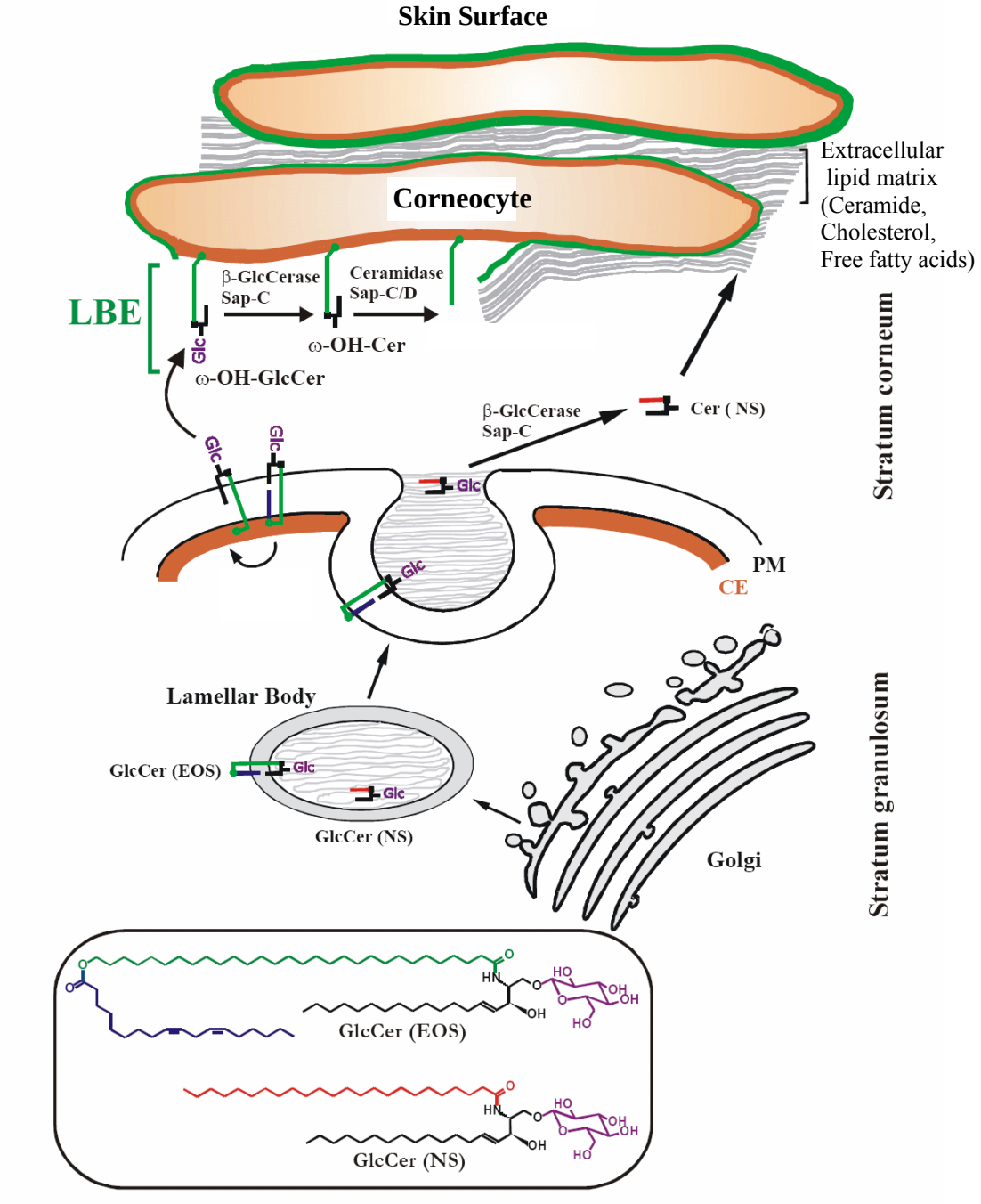

Fig. 5. Extracellular metabolism of glucosylceramides (GlcCer) and genesis of the lipid bound envelope (LBE, green) (modified after (Schuette et al. 1999). In the *stratum granulosum*, the lamellar bodies containing glucosylceramides are formed. The ω-hydroxylated and ester bound GlcCers reach through the surrounding membrane of the lamellar bodies. When they fuse with the plasma membrane, the ω-OH GlcCers are attached to the cornified envelope (orange), this process requiring the cleavage of the Linoleic acid residue. At the *stratum granulosum-stratum corneum* interspace, the protein-bound, as well as the free GlcCers are deglycosylated to their respective ceramides through the action of β-Glucocerebrosidase and Sap-C

Lipid barrier ceramides originate in large part from their *probarrier* lipids, glucosylceramides (Uchida et al. 2000) and sphingomyelins (Hanley et al. 1997), by the action of lysosomal hydrolases, namely β-glucocerebrosidase (Holleran et al. 1993) and acid sphingomyelinase (Schmuth et al. 2000). The postsecretory processing of lamellar body derived glucosylceramides to ceramides requires the action of β-glucocerebrosidase (Holleran et al. 1994) and sphingolipid activator protein saposin C (Doering et al. 1999). *Probarrier* lipids and the hydrolytic enzymes are secreted by differentiating keratinocytes at the *stratum granulosum-stratum corneum* interphase. After leaving the basal layer, suprabasal keratinocytes change their gene expression substantially. Previous studies of these changes so far used mainly antisera to proteins or *in situ* hybridization of mRNA with specific radiolabels (Sun and Green 1976). Proteins such as keratins (Roop et al. 1987; Teumer et al. 1994), involucrin (Rossi et al. 1998; Li et al. 2000), profilaggrin (Asselineau et al. 1990) and filaggrin (Steinert et al. 1981), as well as enzymes such as glucosylceramide synthase (Watt 1989; Watanabe et al. 1998) and β-glucocerebrosidase (Holleran et al. 1992), have been primarily used as markers of epidermal differentiation. Recently, attention has increasingly focused on the study of ceramide metabolism, because of its proposed key role in cell differentiation and barrier homeostasis.

2.4.3. The signalling function of ceramides

Functionally, ceramide, excluding ceramide with very long chain fatty acids, has been proposed as a "coordinator" of eukaryotic stress responses (Hannun 1996). This paradigm is supported by the repeated findings that many inducers of stress response (not limited to those inducing apoptosis) result in ceramide accumulation, usually as a result of activation of either SMases (Andrieu-Abadie et al. 2001) or the *de novo* pathway (Perry 2000), but sometimes as a result of inhibition of ceramide clearance through sphingomyelin synthase or ceramidase. These inducers include cytokines (TNF, Fas, nerve growth factor), environmental stresses (heat, UV radiation, hypoxia/reperfusion), chemotherapeutic agents (doxorubicin, etoposide, and many others), and other miscellaneous agents (dexamethasone, lipopolysaccharide, sitosterol, and B-cell receptor stimulation). Several lines of investigation now support roles for endogenous ceramide in mediating/regulating many key and specific cellular responses. For example, inhibitors of GCS and of ceramidases have been shown to induce increases in ceramide levels and cause apoptosis and/or cell cycle arrest, especially of cancer cells, and overexpression of bacterial SMase has been shown to induce apoptosis and cell cycle arrest (Hannun et al. 2001). If accumulation of ceramide through de novo biosynthesis,

sphingomyelin degradation, or reduced GlcCer synthesis, leads to apoptosis, the pathway through sphingosine and sphingosine-1-phosphate leads to the opposite effect, mitogenesis and increased proliferation. This pathway is activated through increased hydrolysis of ceramide by ceramidase. Thus, ceramide and sphingosine, through their phosphorylated form, act as a two component switch which, dependently on the modulators present in the medium, lead the cells to either apoptosis (ceramide and sphingosine) or proliferation (ceramide-1-phohsphate and sphingosine-1-phosphate). However, it must be kept in mind that only ceramide, and not its phosphorylated form ceramide-1-phosphate, leads to apoptosis. The phosphorylated form acts like sphingosine-1-phosphate as a mitogene agent, albeit through a different signalling pathway.

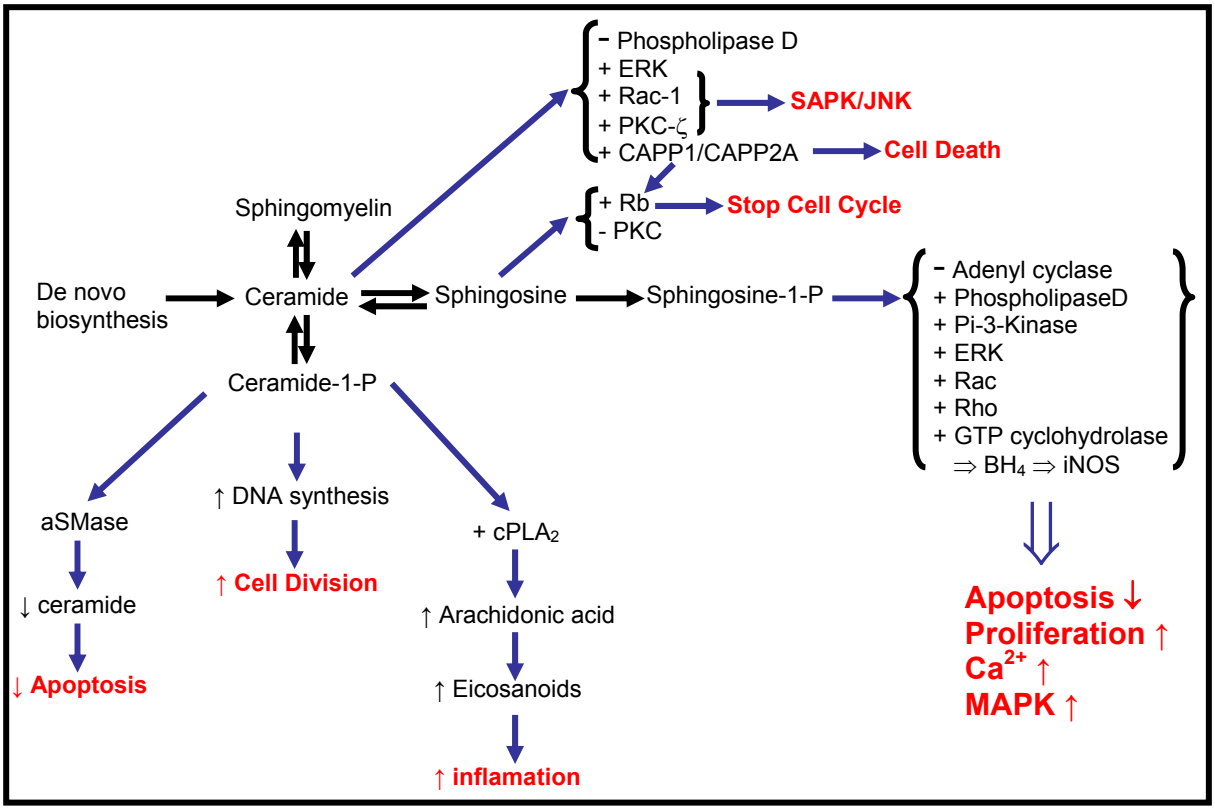

Fig. 6. Ceramide as coordinator of eukaryotic cell stress response. Ceramide signalling can be achieved through ceramide itself (proapoptotic), or its phosphorylated form ceramide-1-P or a catabolism product such as sphingosine in its phosphorylated form sphingosine-1-phosphate). Both these phosphorylated forms are mitogenic. **Abbr.** aSMase: acid sphingomyelinase; $cPLA_2$: cytosolic phospholipase A2; CAPPP: okadaic acid -sensitive protein phosphatase; Rb: retinoblastoma; PKC: protein kinase C; ERK: extracellular signal regulated kinase; BH_4: tetrahydrobiopterin; MAPK: mitogenic activator of protein kinase.

2.5. Nitric oxide

In recent years, it has been suggested that increased nitric oxide levels (NO) favour keratinocyte proliferation, whereas reduced NO levels might correlate with keratinocyte differentiation (Bruch-Gerharz et al. 1998) (Rossi et al. 2000). NO is implicated in several signaling pathways related to a diverse array of cell functions (Krischel et al. 1998). It is especially well known as a mediator of blood vessel relaxation (Moncada and Higgs 1993; Billiar 1995) and has been implicated in the protective immune response by its cytotoxic activity in macrophages that helps to defeat microbes and tumor cells (Liew and Cox 1991). NO action is mediated by fast acting signalling cascades, which rapidly subside after the disappearance of the original stimulus (*e.g.* calcium influx). NO can also stimulate signaling pathways that elicit long lasting changes in the cell, and it can also reset genetic programs.

2.5.1. Nitric oxide synthesis

NO synthesis is catalyzed in mammals by Nitric Oxide Synthase (NOS), a flavoheme-containing enzyme (Rudolf et al. 2002) (Hendrich et al. 2002). NOS needs NADPH and O_2 to hydroxylate a guanidino nitrogen of L-arginine and then oxidizes the N^{ω}-hydroxy-L-arginine intermediate (NOHA) to NO and citrulline. Three isoforms of NO have been described : two constitutively expressed isoforms (endothelial eNOS and neuronal nNOS) and one inducible isoforms (iNOS) expressed mainly in inflammatory and immune cells (Nathan and Xie 1994; Zamora et al. 2000). These enzymes share ~50% sequence homology and are active only as a dimer. Each monomer has a C-terminal diflavin-reductase domain and an N-terminal oxygenase domain. Dimerization is thought to activate the enzyme through sequestering of iron, generating high-affinity binding sites for arginine and the essential cofactor tetrahydrobiopterin (BH4), and allowing electron transfer from the reductase-domain flavins to the oxygenase-domain heme. Activity is also dependent on bound calmodulin (Alderton et al. 2001). In iNOS, calmodulin is tightly bound, whereas in eNOS and nNOS, calmodulin binding is dependent on calcium, and enzyme activity is therefore calcium dependent. Bound calmodulin is thought to enhance the rates of electron transfer through the reductase domain to the oxygenase domain. In addition to regulation of NO synthesis by cofactors and calcium, the activities of NOSs can be altered by post-translational modifications and by protein–protein interactions. For example, eNOS activity is increased by phosphorylation of serine at position 1179 (McCabe et al. 2000), and is inhibited by interaction with the scaffolding domain of the membrane protein caveolin-1.

The first described physiological target for NO was soluble guanylyl cyclase (Ignarro et al. 1999). Binding of NO to the iron within the heme moiety of guanylyl cyclase produces a conformational change that leads to enzyme activation. The subsequent rise in cyclic GMP accounts for many of the physiological effects of NO. However, NO has the potential to interact directly or indirectly with metals, thiols and oxides, and affect proteins, nucleic acids, lipids and sugars (Davis et al. 2001). Because NO is a free radical, and the nitrogen can exist in various oxidation states to generate nitroxyl ions (NO^-), NO free radicals ($NO^\bullet$), nitrosonium cations (NO^+), nitrite ions (NO_2^-) or nitrate ions (NO_3^-), the biological chemistry of NO is complex, and its potential effects within biological systems are many. There are probably four main targets for reactions with NO in cells: metals, reduced thiols, molecular oxygen and other reactive oxygen species (for example, superoxide radical anion ($O^\bullet{}_2^-$)). Which reactions are favoured will depend on the concentration of the reactants and the reaction rates (Gow and Ischiropoulos 2001).

2.5.2. Nitric oxide signalling

NO can promote or inhibit apoptosis (Moncada and Erusalimsky 2002), kill tumours or increase the potential for metastasis or vascularization (Lala and Chakraborty 2001), increase or protect against damage after stroke (Huang 2000) (Dawson and Dawson 1998) and trigger ischaemic preconditioning in the heart or mediate late effects of preconditioning (Dawn and Bolli 2002). Some of these dual effects of NO relate to different isoforms of NOS regulating different processes. For example, protective effects in stroke seem to be mediated by eNOS (vascular), whereas harmful effects are due to nNOS activity (neuronal toxicity) (Huang et al. 1994) (Dawson and Dawson 1998). Other dual effects might relate simply to the amount of NO generated or the background redox state of the cell; at low concentrations, NO seems to be anti-apoptotic, in part through inhibition of caspase activity by means of nitrosation, whereas at higher concentrations, it can indirectly activate caspases (Kim et al. 2002). The dual effects of NO are difficult to study as beneficial effects can be offset by harmful effects or turn into harmful effects, depending on the underlying rates of NO synthesis.

2.5.3. Nitric oxide modulation

In most cell types, exogenously added NO generating compounds such as sodium nitroprusside, S-nitroso-N-acetyl-D,L-penicillamine (SNAP) and S-nitrosoglutathion have been reported to induce apoptosis, e.g. in human leukemia HL-60 cells (Kuo et al. 1996) and in macrophages (Albina et al. 1993; Messmer and Brune 1996), along with ceramide

generation from either the *de novo* or sphingomyelin degradation pathways (Huwiler et al. 1999). On the other hand, NO added exogenously in concentrations less than 0.25 mM can increase proliferation of human epidermal keratinocytes (Krischel et al. 1998). This is in accordance with observations that the process of epidermal wound repair is associated with a high induction of iNOS (Stallmeyer et al. 1999). The availability of NO is thought to cause a hyperproliferative state, whereas reduced NO levels may correlate with differentiation of keratinocytes (Krischel et al. 1998). Although the molecular details are mostly unknown, the effects of NO seemed to be indirect (Bogdan 2001), through modulation of transcription factors such as NF-κB (Matthews et al. 1996) and AP-1 (Tabuchi et al. 1994). In a previous study, Döring studied the effect of NO on the ceramide content of differentiated keratinocytes (Döring 1999). He was able to establish a correlation between the NO concentration and the amount of generated ceramide. As seen in Fig. 7, the higher the NO concentration, the lower the ceramide content, and vice versa.

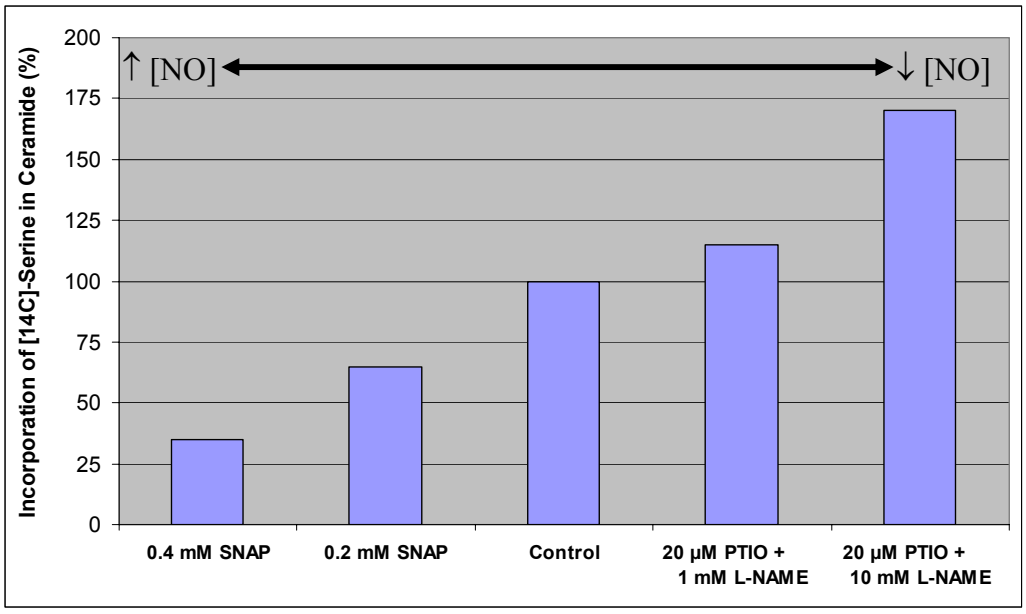

Fig 7. Effect of modulators of cellular NO concentration on the biosynthesis of ceramide in differentiating keratinocytes. Human epidermal keratinocytes were cultivated in low calcium medium until confluence, then shifted to high calcium medium for 7 days. Cells were incubated with the indicated NO modulators for 24 hours. The ceramides were labelled with [3-^{14}C]-serine, extracted and quantified using a phosphoimager (see materials and methods). Abrv. SNAP: S-nitrosylpenicillamine; PTIO: 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3 oxide; L-NAME: L(omega)-nitro-arginine methyl-ester.

All these finding point to the important role played by NO in the proliferation and differentiation of keratinocytes, through its ability to interact with ceramide and the different components of its metabolism.

3. Results

3. Results

3.1. mRNA expression profiles of cultured keratinocytes under different conditions.

3.1.1.1.Optimization steps for the Real-Time quantitative PCR

Real Time quantitative PCR (RTQ-PCR) is a very sensitive method to measure the mRNA amounts of expressed genes. It is based on the PCR principle and necessitates for every measured mRNA 2 primers as well as a fluorescent probe. These Primers-Probe triplets need to be optimized for every measured target gene. Table 1 lists all primer-probe sets for all measured genes as well as the optimized amplification conditions (forward and reverse primer concentrations vary between 50 and 900 nM) for each one. The optimal $MgCl_2$ concentration varies between 3.5 and 6 mM. Porphobilinogen deaminase (PBGD) was used as a house keeping gene to normalize the amounts of RNA used in the experiments. Its expression was measured in keratinocytes and fibroblasts in different culture conditions and was found to have a constant expression.

Tgt	Forward Primer	[nM]	Reverse Primer	[nM]	Probe
Ker 14	GGCTTTGCTG GTGGTGATG	**900**	CTGCCTCTGGT ACCAGTCACG	**300**	CCATGCAGAACC TCAATGACCGCC
Ker 10	ATAATGCCAAC ATCCTGCTTCA	**900**	AGGCCGTTGA TGTCAGCCT	**900**	GTCATCAGCTGCC AGCCTTGCATTG
ACerase	TGAACTCGATG CTAAGCAGGGT	**900**	GGTGCGGTTCA GACACATCTT	**300**	TCTTCCTTGATGA TCGCAGAACGCC
aSM	GGCACTGTCT GAAGAGCTGG	**300**	AGAAGACCTCA AATTCATCCACAT	**900**	ACCGAATTGTAGCCA GGTATGAGAACACC
GCS	GGTCCGCAACT TTGTCGATAG	**900**	CCACCAACGC CACTCTCTG	**900**	GTGCCCGTGTGA TTAGCCTGGATGG
β-GCase	CATATGGTGC CCAACCCTG	**900**	GGGTCAAGAGA GTCACAGTACGA	**900**	CCTCCAACCTCG GTCAGCTATCGCTT
SPT LC2	GGAGATGCTG AAGCGGAACA	**300**	GGTATGAGCTG CTGACAGGCA	**900**	CAGGAAATCCAAC CACAACGACACCG
pSAP	TTGCCAGGAC TGCATTCAGAT	**300**	CCACCAAGG CCTGGACAA	**900**	TGGTCCGTACAGC AGTCTGGATGTCAG
pFG	CCATCATGG ATCTGCGTGG	**900**	CACGAGAGGA AGTCTCTGCGT	**900**	GAGTGCCCATGAC TGGCTCTGTCTTCA
PBGD	CTGCACGATC CCGAGACTCT	**900**	CCTCCAGTCAG GTACAGTTGCC	**300**	CTGCAGTGTGCCA GTAGCCGTGCATA

Table 1: List of used Primer pairs and probes for the Real time quantitative PCR assays. All nucleotides are listed from 5' to 3' from left to right. Tgt: target gene; [ ]: concentration; Ker 14: keratin 14; Ker 10: keratin 10; aCerase: acid ceramidase; aSM: acid sphingomyelinase; GCS: glucosylceramide synthase; β-GCase: β-glucocerebrosidase; SPTLC2: Serine palmitoyltransferase subunit LC2; pSAP: sphingolipid activator protein precursor; pFG: profilaggrin; PBGD: Porphobilinogen deaminase.

In many cases, there is a disconnect between mRNA expression and physiological effect of a gene. For example, an expressed enzyme may well be up-regulated, even though the expressed form remains inactive and has to be posttranslationally modified to become active.

In order to be able to analyze the significance of the expression of genes measured by RTQ-PCR, it is necessary to establish a correlation between the measured mRNA levels and the metabolic effect of the measured target, such as enzyme activity. We measured the enzyme activity of serine palmitoyltransferase and β-glucocerebrosidase (Fig 8), as well as made western blots for keratin 10 (data not shown). Basically, keratin 10 protein levels paralleled their respective mRNA levels during keratinocyte differentiation. We found a similar correlation for the two enzymes, serine palmitoyltransferase and β-glucocerebrosidase. We and other groups provided similar correlations for other enzymes such as acid sphingomyelinase (Langmann et al. 1999), acid ceramidase (Maeda et al. 1999) and glucosylceramide synthase (Watanabe et al. 1998).

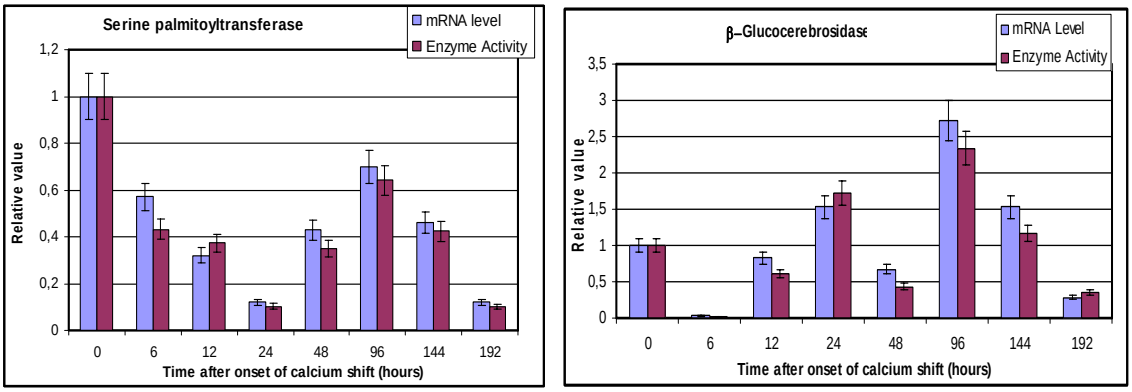

Fig. 8. mRNA level and enzyme activity of serine palmitoyltransferase and β-glucocerebrosidase during calcium shift. The enzyme activity of β-glucocerebrosidase at timepoint 0 hours is 2.48 ± 0.27 nmol/min/mg of protein. The enzyme activity of serine palmitoyltransferase at the same timepoint is 163 ± 22 pmol/min/mg of protein.

3.1.2. Expression pattern of proliferating keratinocytes

Keratinocytes were cultivated for 8 days in MCDB-153 medium with 0.1 mM Ca^{2+} (low calcium). In this medium, keratinocytes are in a proliferating stadium. The nucleus of these cells has a cylindrical form, with high heterochromatin content. The cells in low calcium form a monolayer and are assimilated as being in a stratum basale state. Total RNA was extracted at regular intervals (0, 1, 3, 5, and 8 days of culture), and the mRNA of different proteins involved in keratinocyte metabolism were measured by real-time quantitative PCR (RTQ-PCR). The profile of these cells shows that Keratin 14, a marker of the *stratum basale*, is expressed during the complete phase, indicating that the cells are in a proliferative state (Fig. 9). This is corroborated by the absence of expression of both Keratin 10 and profilaggrin, two proteins expressed by differentiating keratinocytes (Fig. 9).

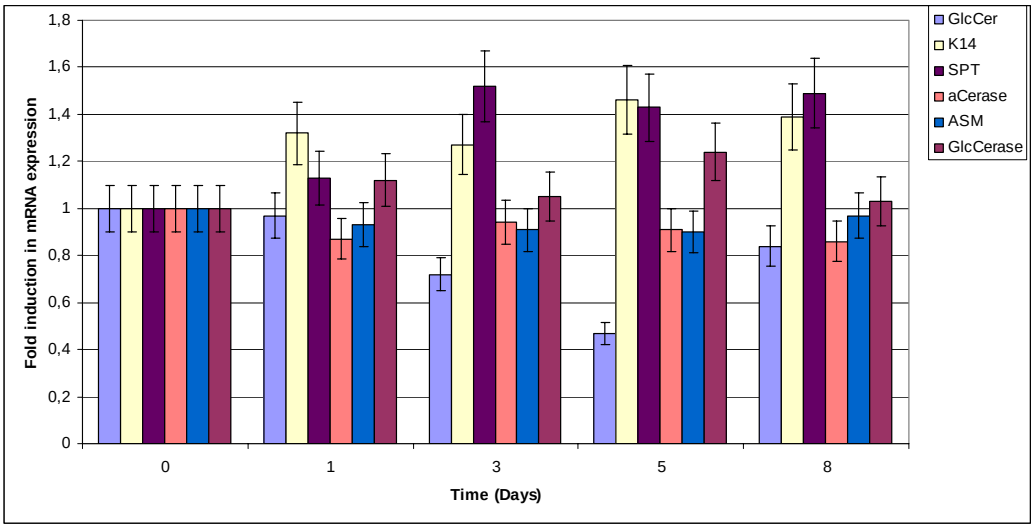

Fig. **9. Expression profile of proteins and enzymes of the ceramide metabolism in proliferating keratinocytes.** Keratinocytes are cultivated in low calcium MCDB-153 medium and cells were harvested at the indicated time points. Total RNA was extracted and measured with RTQ-PCR. GlcCer: glucosylceramide synthase; K14: keratin 14; SPT: Serine palmitoyltransferase subunit LC2; aCerase: acid ceramidase; ASM: acid sphingomyelinase; GlcCerase: β-glucocerebrosidase.

3.1.3. Expression pattern of differentiating keratinocytes

Human epidermal keratinocytes (HEK) were cultivated to confluence in low calcium medium and then shifted to high calcium. Total RNA was extracted at the indicated time points and the mRNA for the different proteins and enzymes of the sphingolipid metabolism were measured by RTQ-PCR. The mRNA levels of enzymes involved in the ceramide metabolism show an increase in expression compared to time point 0 (calcium shift, start of differentiation). The increase is more pronounced for acid sphingomyelinase, though this increase takes place at day 8 (192 hours, Fig 10A).

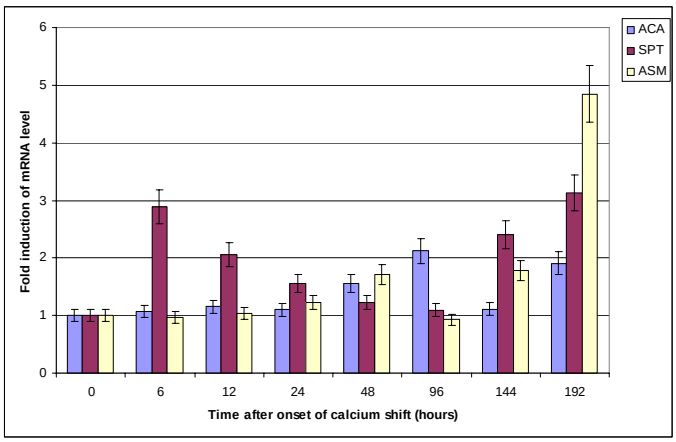

Fig 10A. mRNA expression levels of enzymes of the ceramide metabolism in differentiating keratinocytes. Keratinocytes are cultivated in low calcium MCDB-153 medium and then shifted to high calcium at time point 0 hours. Cells were harvested at the indicated time points. Total RNA was extracted and measured with RTQ-PCR. ACA : acid ceramidase; SPT: Serine Palmitoyltransferase; ASM: acid Sphingomyelinase.

The enzymes glucosylceramide synthase and β-glucocerebrosidase also show an important increase toward the end (144 and 192 hours of culture, Fig. 10B). This is consistent with their proposed role in the establishment of the permeability barrier. As both enzymes are antagonists, it appears that the net effect of this increase is minimal. However, both these enzymes are active in different compartments (glucosylceramide synthase at the Golgi membrane, β-glucocerebrosidase in the extracellular space of the *stratum corneum*). Thus, these enzymes become synergistically active, and their net effect is the establishment of the lipid barrier in the *stratum corneum*.

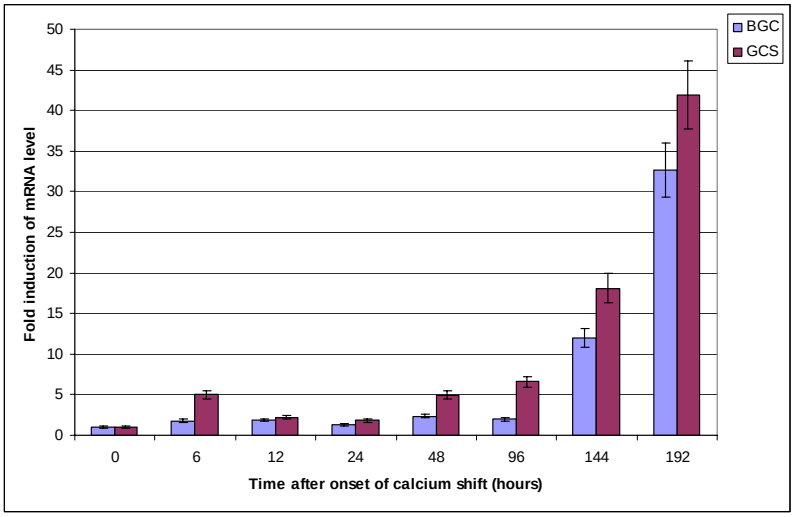

Fig 10B. mRNA expression levels of glucosylceramide synthase (GCS) and β-Glucocerebrosidase (β-GC) in differentiating keratinocytes. Keratinocytes are cultivated in low calcium MCDB-153 medium and then shifted to high calcium at time point 0 hours. Cells were harvested at the indicated time points. Total RNA was extracted and measured with RTQ-PCR. BGC: β-Glucocerebrosidase; GCS: Glucosylceramide Synthase.

The calcium shift induces changed in the expression profiles of other proteins as well. For example, as the mRNA level of Keratin 14 becomes below detection limits, that of Keratin 10 and profilaggrin, previously undetectable, increase dramatically, with Keratin 10 seeming to be one of the first targets of the calcium shift (increase 30 fold after 6 hours of induction, Fig. 10C). The increase in profilaggrin expression toward the end is explained by its role in building the protein scaffold of the permeability barrier. The increase in pSAP is also consistent with its role in the genesis of the lipid barrier, as it is a necessary element for the barrier building enzymes such as β-glucocerebrosidase.

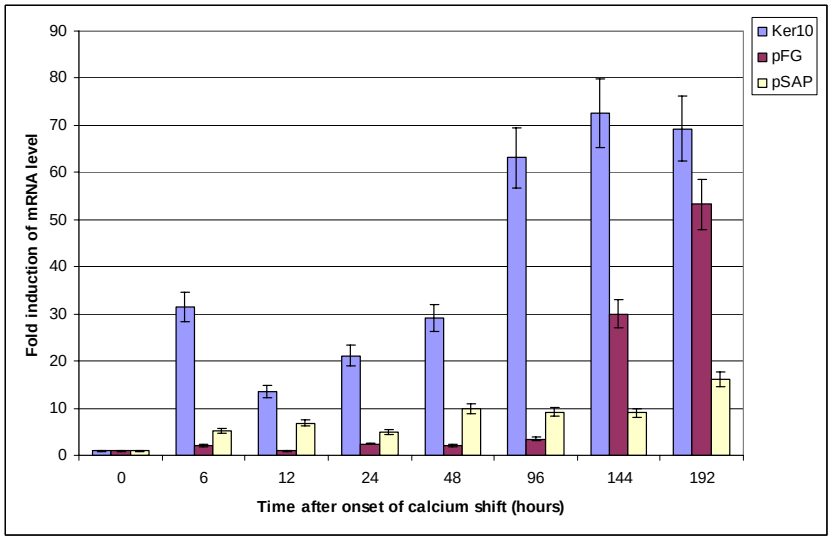

Fig 10C. mRNA expression levels of markers of differentiation of keratinocytes. Keratinocytes are cultivated in low calcium MCDB-153 medium and then shifted to high calcium at time point 0 hours. Cells were harvested at the indicated time points. Total RNA was extracted and measured with RTQ-PCR. Ker10: keratin 10; pFG: profilaggrin; pSAP: prosaposin. Ker10: Keratine 10; pFG: Profilaggrin; pSAP: sphingolipid activator protein precursor.

3.1.4. Effect of vitamin C and linoleic acid on the expression pattern of differentiating keratinocytes

We investigated the effect on the mRNA expression pattern of the addition of 10 µM linoleic acid to the culture medium at the calcium shift (Table 2). The measurements showed that the mRNA levels of all markers increased, compared to cells cultivated in high calcium medium, with the exception of keratin14 which could not be detected.

Marker	**mRNA level Increase** (expressed in % of high calcium without linoleic acid)
SPT	10
β-GCase	20
GCS	40
pSAP	40
pFG	100
Ker10	200
aCerase	250
ASM	300
Ker14	Not detected

Table 2. mRNA expression levels of differentiation markers and enzymes of the ceramide metabolism measured in keratinocytes cultivated in high calcium MCDB-153 medium with 10 µM linoleic acid compared to cells cultivated in high calcium without linoleic acid (used as control). SPT: Serin palmitoyltransferase; β-GCase: β-glucocerebrosidase; GCS: glucosylceramide synthase; pSAP: sphingolipid activator protein precursor; pFG: profilaggrin; Ker10: keratin 10; aCerase: acid ceramidase; ASM: acid sphingomyelinase; Ker14: keratin 14.

3. Results

The analysis of the expression patterns of keratinocytes cultivated at high calcium and linoleic acid with 50 µg/ml vitamin C showed no difference compared to controls without vitamin C (data not shown). The cultures showed a normal expression pattern, similar to that previously described in this work.

3.2. Regulation of Ceramide Biosynthesis during Differentiation of Keratinocytes

We investigated the *de novo* synthesis of ceramides during differentiation of cultured keratinocytes. Confluent cultured human keratinocytes were triggered to differentiate by shifting the medium to a high calcium ion concentration (1.1 mM $CaCl_2$, 10 µM linoleic acid). Within 6 hours of start of the metabolic labeling with L-[3-^{14}C]-serine, they produced mainly ceramide 2 (Cer(NS)) with acyl chains of 16 to 24 carbon atoms as identified by co-migration of TLC standards (Macheleidt et al. 2002) and only minor amounts of complex ceramides. Within 24 hours of calcium shift, the level of labeled ceramide increased up to 175%, and that of glucosylceramide and sphingomyelin up to 137% and 200%, respectively (Fig 11). During the following 7 days of differentiation, *de novo* ceramide synthesis dropped again to about the starting value.

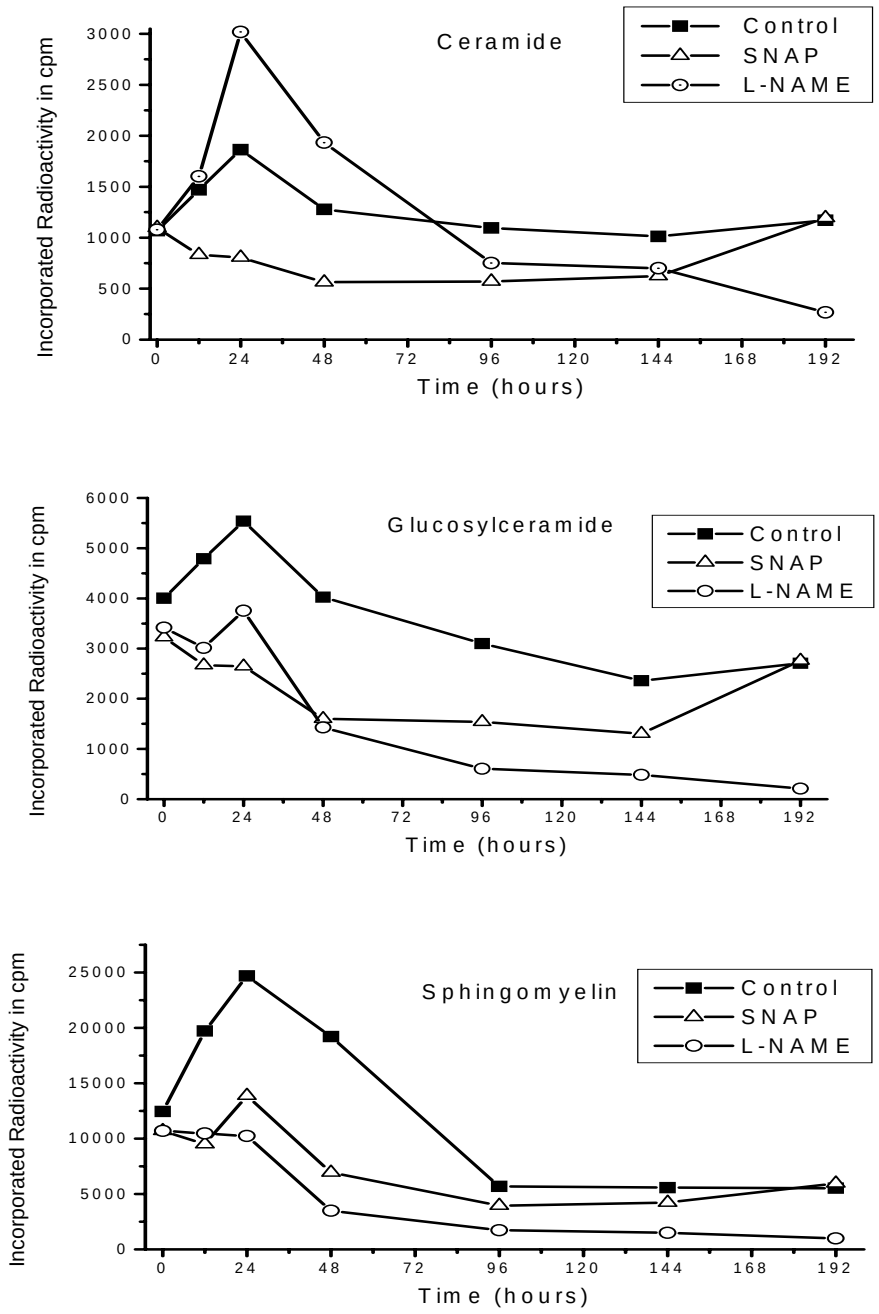

Fig. 11. The *de novo formation* of ceramide during keratinocyte differentiation is modulated by nitric oxide. Human epidermal foreskin keratinocytes were cultivated in triplicates to confluence and differentiated by a high calcium shift for up to 8 days as described under experimental procedures. The cells were harvested at the indicated time points after starting of the calcium shift. 6 h before harvesting, the cells were labeled with L-[3-^{14}C]-serine. Lipids were extracted and analyzed as described. Identical amounts of the lipid samples, relative to cellular DNA amounts, were applied to the TLC plates. The incorporated radioactivity was visualized on thin-layer chromatograms then quantified (referring to the same amount of cellular DNA) by phospho-imager analysis. The amount of radioactivity is given in PSL (photo stimulated luminescence), and analyzed as described under experimental procedures. We analyzed the samples in triplets and assessed the measurement error to be less than ± 5%.

3.3. **Cellular NO Modulation**

Keratinocytes were cultivated in low calcium medium until confluence. At the calcium shift, NO modulators SNAP or L-NAME (N^G-Nitro-L-Arginin-methylester) were added to the medium (see materials and methods). Addition of the NO-donor SNAP to the culture medium induced a reduction in ceramide synthesis down to about 60% of the starting value after 48 hours (Fig. 11). Then, ceramide formation increased slowly and reached again the level of control keratinocytes after 8 days. Compared to untreated keratinocytes, the addition of L-NAME, an inhibitor of iNOS, enhanced ceramide labeling during the first 24 hours after calcium shift to a peak value of 280% of the starting value, followed by a continuous decrease to about 25% of the starting value after 8 days. However, it did not cause a transient increase of the labeled glucosylceramide and/or sphingomyelin levels, the other major sphingolipid components and ceramide precursors in keratinocytes.

3.3.1. **Influence of NO Donor or NOS Inhibitor on Cultured Keratinocytes**

The morphological changes of epidermal keratinocytes and dermal fibroblasts during culture with NO donor or NOS inhibitor were observed with a light microscope.

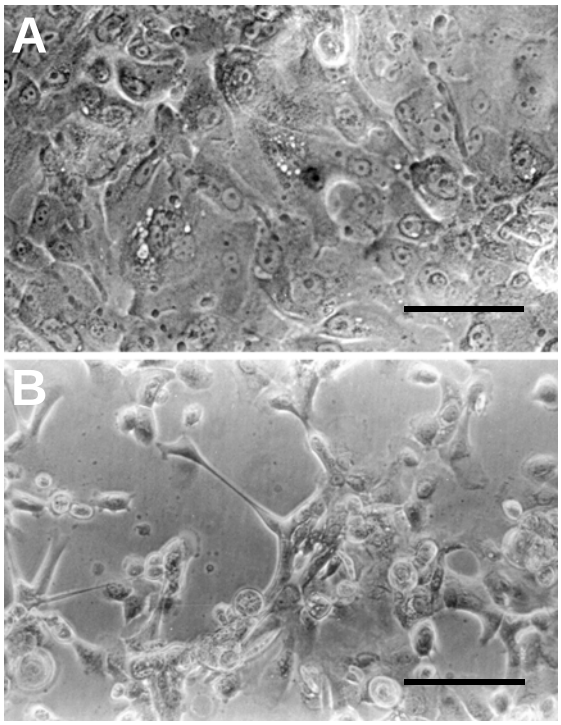

Fig. 12. Effect of calcium shift and nitric oxide on keratinocytes differentiation in MCDB medium. Represented is differentiation stage 6 days after calcium shift. **A**: untreated keratinocytes. **B**: keratinocytes treated with 10 mM L-NAME (see experimental procedures). Bars represent 50 µm.

3. Results

Human keratinocytes were grown under low calcium ion concentration (0.1 mM $CaCl_2$) to confluence as a monolayer of non-stratifying cells (cells at time point 0 days). After shifting to a high calcium concentration (1.1 mM $CaCl_2$, 10 µM linoleic acid) the keratinocytes underwent stratification and changed their shape in the upper cell layer (Fig. 12A). The additional treatment with the NO donor SNAP (0.2 mM) did not change the morphology significantly. Supplementation of the culture medium with the NOS inhibitor L-NAME, however, had a different effect. After 2-3 days, the keratinocytes started to contract, yielding at first stellate cells and later globular cells. No defined nuclei could be observed anymore and many cells sloughed from the dish (Fig. 12B). The sensitivity against the NOS inhibitor seems to be quite specific to epidermal keratinocytes since dermal fibroblasts showed a different behavior. Human fibroblasts showed a reversed morphology after treatment with SNAP or L-NAME. Addition of the inhibitor L-NAME induced no visible effect on the fibroblast morphology, whereas the addition of SNAP caused them to contract and slough from the dish (data not shown).

We conducted an apoptosis assay based on the measurement of the fluorescence related to the activity of caspase-3 (see materials and methods). This assay showed that keratinocytes treated with SNAP had an apoptotic activity comparable to that of untreated cells. On the other hand, the addition of L-NAME clearly induced apoptosis (Fig. 13). The picture was completely reversed in fibroblasts, as only SNAP, but not L-NAME, induced caspase-3 activity, and ultimately apoptosis.

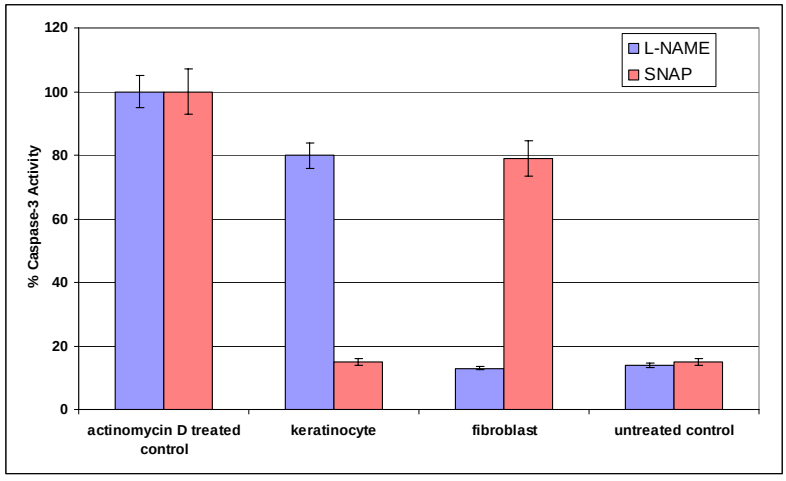

Fig. 13. Apoptosis in cultured keratinocytes and fibroblasts. Caspase-3 activity was measured in keratinocytes and fibroblasts, which had been cultivated for 7 days in the calcium shift medium containing either SNAP (0.2 mM) or L-NAME (10 mM) as described under experimental procedures. Values represent the mean of triplicates and are expressed relative to caspase-3 activity measured in the respective cell types after treatment with 50 µM actinomycin D that induces apoptosis (positive control set as 100%). Untreated keratinocytes and fibroblasts have been used as negative control for both cell types.

3.3.2. **Calcium Shift Increases mRNA Levels of Differentiation Markers**

As previously shown by northern analysis, mRNA transcription of keratin10, profilaggrin and other suprabasal differentiation markers is induced in cultured keratinocytes by the calcium shift (Roop et al., 1987). In order to quantify these changes, the mRNA levels of keratin 10 (Fig. 14A), keratin 14 (Fig. 14B) and profilaggrin (Fig. 14C) were analyzed at the beginning of the differentiation period using RTQ-PCR (see experimental procedures). The data obtained during the calcium shift confirm previous reports that keratin 10 is a good differentiation marker of keratinocytes as its mRNA expression increased about 30-fold within the first 6 hours after calcium shift, then decreased by about 50 % after 12 hours and reached a peak of a 70-fold increase after 6 days of differentiation. The expression rate of profilaggrin increased up to 4-fold during the first 4 days of calcium shift, and then increased dramatically 55 fold after 8 days (Fig. 14C). In contrast, the mRNA levels of the basal marker keratin 14 remained basically unchanged throughout the entire differentiation time (Fig. 14B). It is interesting to note that mRNA levels of the above mentioned proteins except keratin 14 increased noticeably already within the first 6 hours of the calcium shift. Similar observations were made with mRNA levels of several proteins involved in sphingolipid metabolism, e.g. glucosylceramide synthase (Fig. 15A), serine palmitoyltransferase, (Fig. 15C), and prosaposin (Fig. 15D). After calcium shift, mRNA levels of glucosylceramide synthase, β-glucocerebrosidase and prosaposin (pSAP) increased continuously for more than an order of magnitude (Fig. 15) to reach their maximum after 8 days, whereas human acid ceramidase and acid sphingomyelinase reached a rather low maximum already after 4 days (Fig. 15E and 15F). mRNA levels of prosaposin (Fig. 15D) increased five fold within the first 6 hours and then increased further up to 16-fold of the starting value after 8 days. In contrast to these observations, the expression levels of serine palmitoyltransferase, the rate-limiting enzyme of sphingolipid *de novo* biosynthesis, increased only up to 3-fold after onset of the calcium shift and remained largely unchanged throughout further differentiation (Fig. 15C).

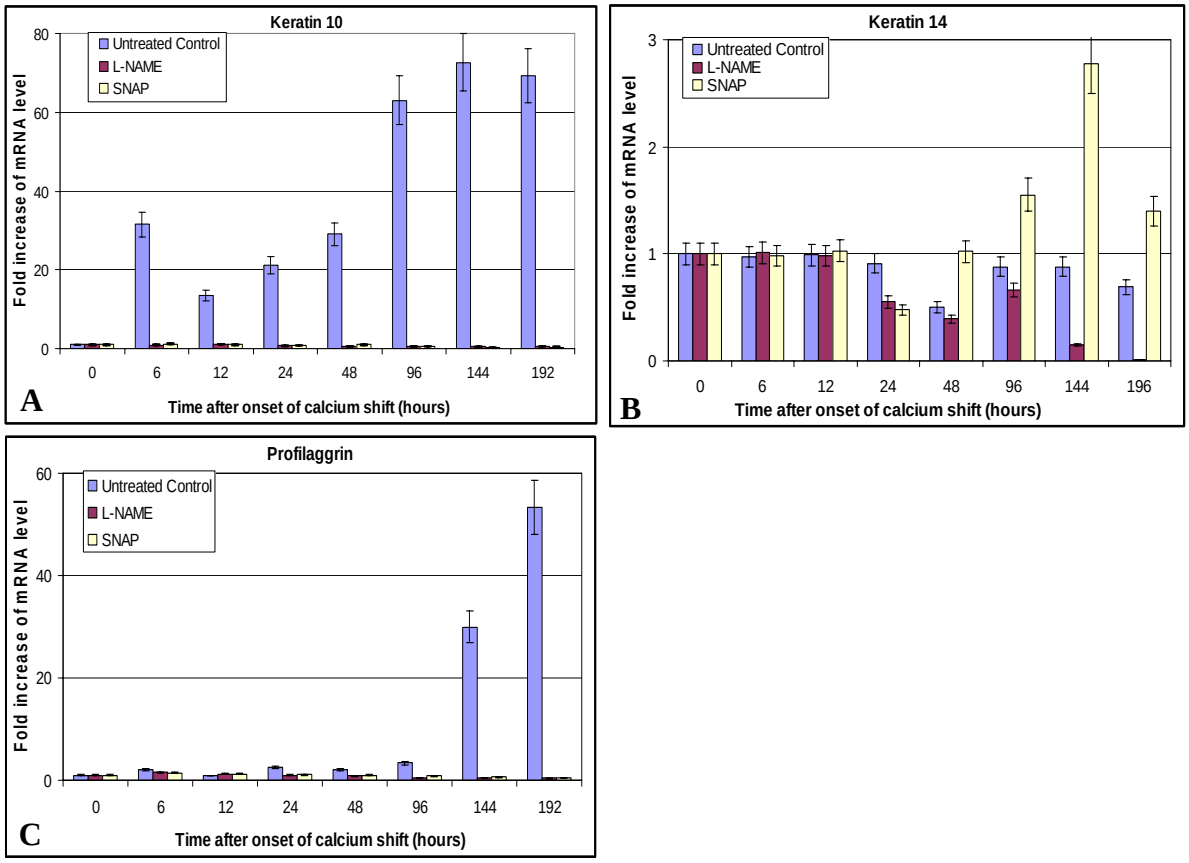

Fig. 14. Expression levels of protein markers in keratinocyte differentiation. Keratinocytes were cultivated in low calcium medium to confluence then shifted in high calcium medium (1.1 mM Ca^{2+} + 10 µM Linoleic acid), the cells were harvested, the total RNA was extracted and the mRNA of keratin 10 (A), keratin 14 (B) and profilaggrin (C) was measured using RTQ-PCR.

3.3.3. Modulators of Nitric Oxide Regulate the Transcription of Enzymes Involved in the Ceramide Metabolism of Cultured Human Keratinocytes

The addition of the NO donor SNAP (0.2 mM) to keratinocyte culture medium under calcium shift conditions essentially did not alter the expression rate of either acid ceramidase (Fig. 15E) or acid sphingomyelinase (Fig. 15F) for the first 12 hours. However, starting after 2 days, their expression rate dropped steadily to about 50 % of that of untreated keratinocytes after 8 days. A similar development was observed after addition of L-NAME, although the drop was down to 8% of that of untreated cells after 8 days, coinciding with apoptosis. The treatment of keratinocytes with SNAP (0.2 mM) or L-NAME (10 mM) affected the mRNA levels of keratin 14 quite differently. After 24 hours, keratin 14 mRNA levels of cells treated with SNAP or L-NAME were lower than those of untreated control cells (Fig. 14B). This development changed after 2 days as the mRNA levels increased in the presence of SNAP until day 6 to almost 3-fold of the control, whereas the mRNA levels assayed in the presence

of L-NAME decreased after day 4 to below the detection limit, coinciding with the onset of apoptosis. The addition of either SNAP or L-NAME resulted in dramatic decreases of the mRNA levels of marker proteins of differentiation, keratin 10 (Fig. 14A) and profilaggrin (Fig. 14C), as well as of proteins crucial for ceramide metabolism, glucosylceramide synthase (Fig. 15A), β-glucocerebrosidase (Fig. 15B), serine palmitoyltransferase (Fig. 15C), and prosaposin (Fig. 15D).

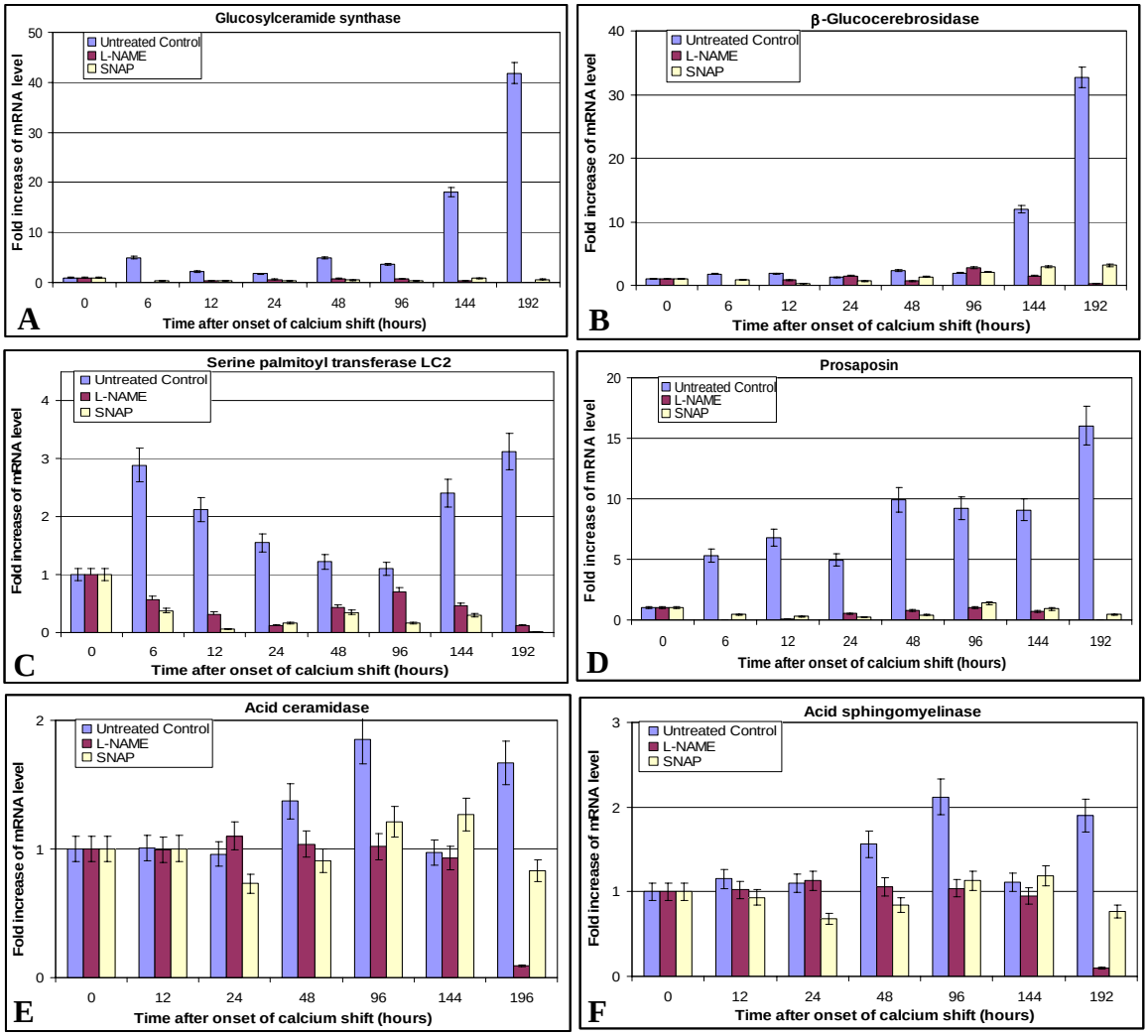

Fig. 15. Expression levels of enzymes of ceramide biosynthesis in differentiating keratinocytes. Keratinocytes were cultivated in low calcium medium to confluence then shifted in high calcium medium (1.1 mM Ca^{2+} + 10 µM Linoleic acid), the cells were harvested, the total RNA was extracted and the mRNA of glucosylceramide synthase (A), β-glucocerebrosidase (B), Serine palmitoyltransferase (C), prosaposin (D), acid ceramidase (E), and acid sphingomyelinase (F) was measured using RTQ-PCR.

3.3.4. Effect of Cellular NO Modulators on the Transcription of Enzymes Involved in Ceramide Metabolism of Cultured Human Fibroblasts

The expression rate of acid ceramidase remained essentially stable in cultured fibroblasts for the first 2 days, and then increased gradually up to 8-fold to peak after 6 days (Fig. 16A). Treatment of fibroblasts with L-NAME increased the mRNA level more than 3-fold compared to the untreated control (and 25-fold compared to the starting value), and with SNAP 3-fold, and 20- fold respectively, compared to the starting value, after 6 days. The mRNA level of acid sphingomyelinase increased up to 12-fold after 6 days of culture (Fig. 16B). This was also true for SNAP treated cells, except that the increase started only after 4 days. In L-NAME treated cells, this increase was reduced by more than 50%. Interestingly, we did not observe a reduction of expression level of both enzymes in SNAP stimulated fibroblasts after 6 days, even though we measured an elevated caspases-3 activity and therefore the onset of apoptosis.

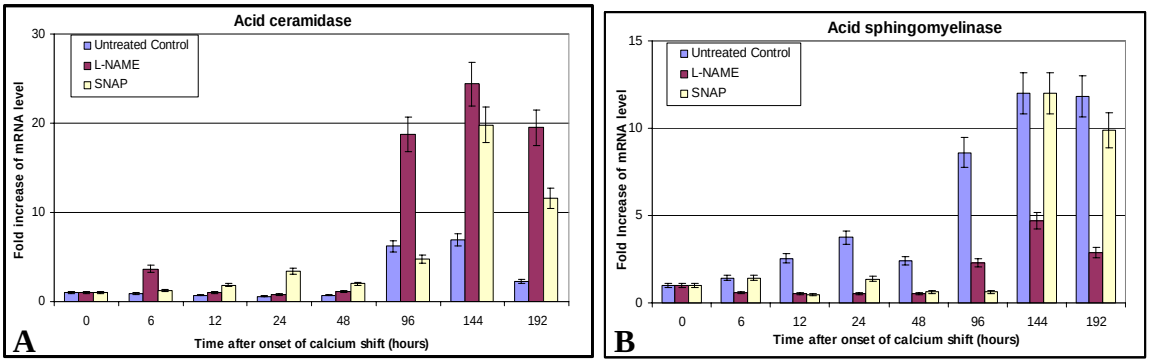

Fig. 16. Expression levels of enzymes of ceramide biosynthesis in fibroblasts. Fibroblasts were cultivated, the cells were harvested, the total RNA was extracted and the mRNA of acid ceramidase (A) and acid sphingomyelinase (B) was measured using RTQ-PCR.

3.3.5. Determination of the origin of ceramides synthesized after addition of L-NAME.

Many studies have linked short chain ceramides with signaling pathways leading to apoptosis. Ceramides are even seen in this respect as having a second messenger function (Zhou et al. 1998; Bieberich et al. 2000; Uchida et al. 2003). (Uchida et al. 2003) demonstrated through metabolic studies that UVB induced apoptosis in keratinocytes was induced by an increase in *de novo* synthesized ceramides (and not sphingomyelin turn over) generated by higher activity of ceramide synthase, and not SPT.

3. Results

In our studies, Keratinocytes treated with the iNOS inhibitor L-NAME deviated from the differentiation program started by the calcium shift, and started an apoptosis program which led to apoptosis 6 days after induction. This coincided with a 280% increase in ceramide synthesis 24 hours after addition of L-NAME. In order to elucidate the nature and origin of the ceramides involved, we did a complex inhibitor study of the metabolic pathways of ceramide. To this effect, keratinocytes were cultivated as previously to confluence in low calcium medium. After the calcium shift, L-NAME was added to the cell culture. We used different radioactive labels, L-[3-^{14}C]-Serine and [3-^{3}H]-Sphingosine. Furthermore, we blocked the different pathways leading to generation of ceramide using Conduritol B epoxid (CBE) or Fumonisin B1 (FB1) separately or jointly (see Fig. 17).

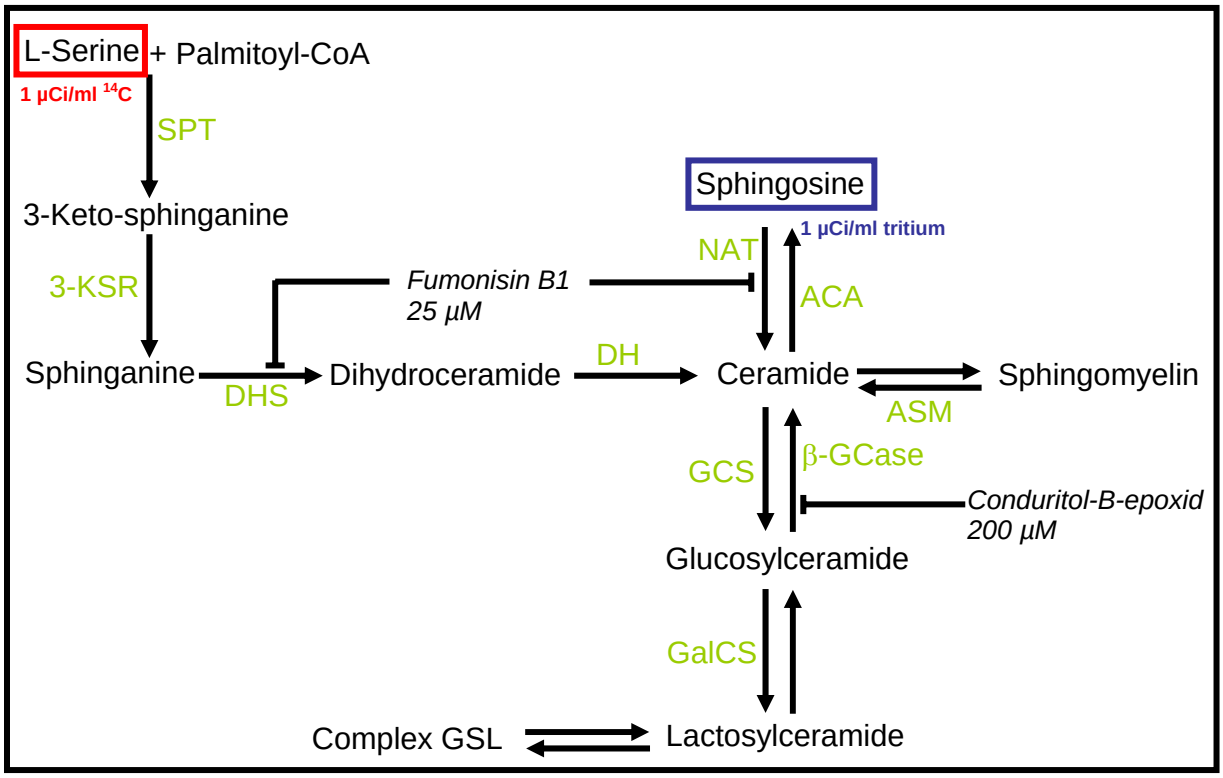

Fig 17. The metabolic pathway of glycosphingolipid biosynthesis with inhibitors.

Cells were harvested at 0 and 12 hours after the calcium shift (see Fig. 18), lipids were extracted and separated on TLC plates, and the lipids were quantified using the phosphor-imager.

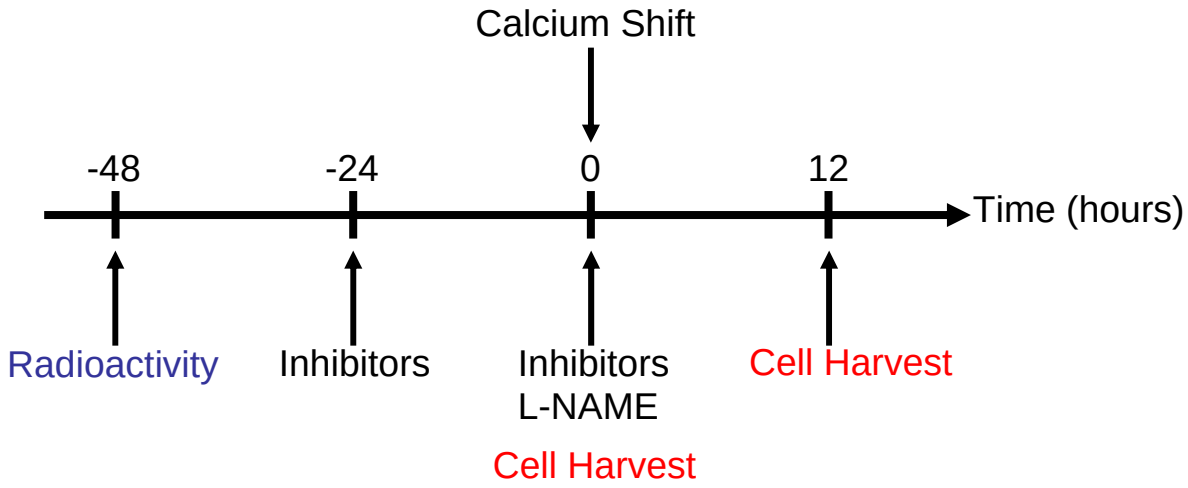

Fig. 18 Experimental time line

The addition of L-NAME to the medium reduced the amount of GlcCer by 30%, whereas the addition of CBE increased GlcCer amounts by 20% (Fig. 19). The concomitant addition of L-NAME and CBE resulted in a GlcCer level equal to that of the control. FB1 did not influence the amount of GlcCer synthesized by the cells, suggesting that they are mainly produced by sphingomyelin turn over and not *de novo* biosynthesis. This is in accordance with the expression measurements described above for differentiating keratinocytes. Furthermore, FB1 did not alter the effect of CBE. The addition of FB1 to cells treated with L-NAME and CBE, however, offset the effect of L-NAME on the inhibition by CBE, as the amount of GlcCer was similar to that of cells treated with CBE alone. This suggests that L-NAME modulates the inhibition by CBE through a reduction of the sphingomyelin turn-over by acid sphingomyelinase, resulting in a reduced biosynthesis of GlcCer. L-NAME increased the amount of Cer(NS) by 40%. This increase is less pronounced (10%) when CBE is added. This suggests that Cer(NS) originates from *de novo* biosynthesis (reduction through FB1 50%), as well as GlcCer (reduction through CBE 40%), with the rest resulting from sphingomyelin turnover (10%). This effect of L-NAME is not generalized to all ceramides since it reduced Cer(NP) by 35% and Cer(EOS) by 20%.

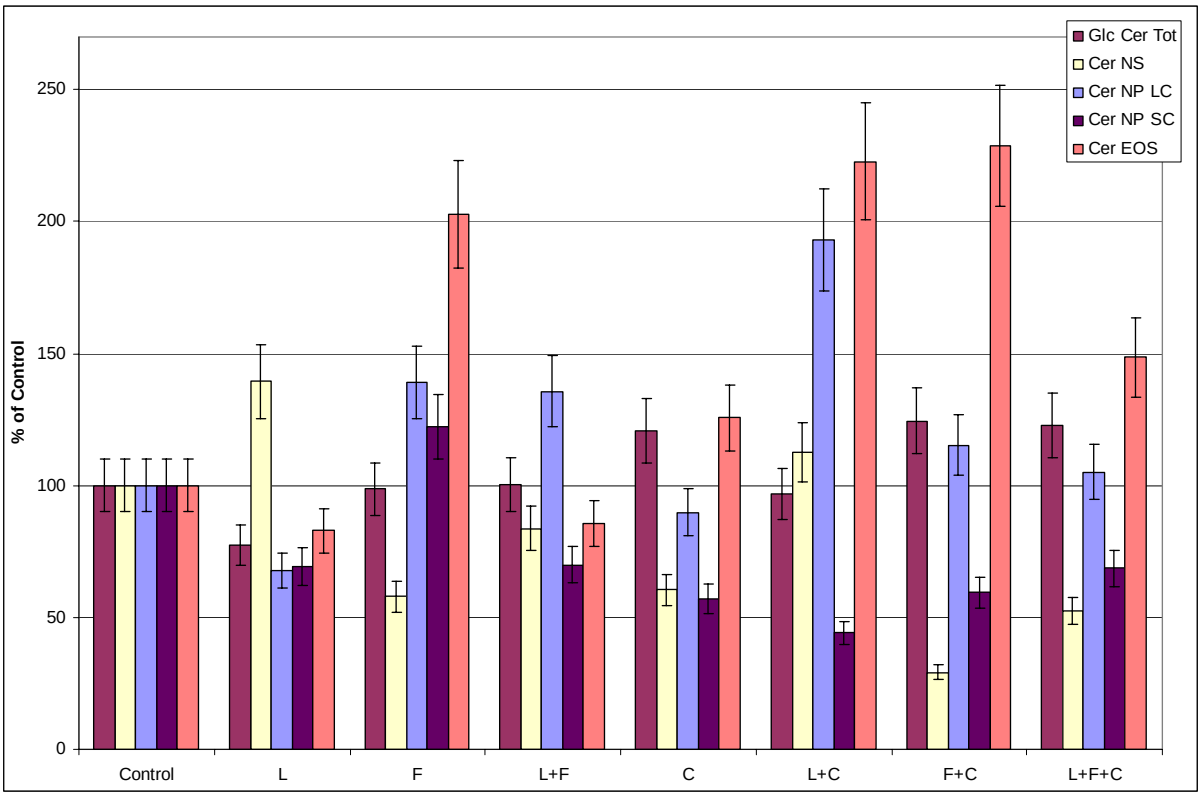

Fig. 19. Amounts of radioactively labeled lipids in differentiating keratinocytes 12 hours after differentiation. The cells were treated with different inhibitors (C: Conduritol B Epoxide; F: Fumonisin B1) and an inhibitor of iNOS (L: L-NAME). After harvest, the lipids were extracted and separated on TLC plates. The radioactivity was visualized by phosphoimager and expressed in percentage of control.

3.4. Analysis of the GCS KO mouse

Morbus Gaucher is a disease caused by a deficiency of β-glucocerebrosidase. The severity of the disease depends on the amount of residual enzyme activity. It is recognized that a residual activity of 5% allow a prolonged life of the patient. However, the absence of this activity results in a lethal disease known as the collodion baby syndrome (Lui et al. 1988), where newborn babies die 1 to 2 days after birth. These babies, among other things, lack a water permeability barrier, and thus continuously loose water through the skin, effectively dying of dehydration. This dramatic phenotype shows the importance of this enzyme. In order to have a better understanding of the genesis of the permeability barrier, we established cooperation with the group of Prof. Gröne (DKFZ, Heidelberg), who has extensive experience with the generation of conditional KO mice.

The GCS KO mouse is a conditional, skin specific, KO mouse. It was established by Richard Jennemann at the DKFZ in Heidelberg and the results of the analysis of the KO mouse are a shared work between both our laboratories.

In order to establish a cell type (epidermis) specific KO mouse of GCS, Richard Jennemann used a promoter of Ker14, which is expressed in basal cells, to drive the Cre-recombinase transgene mouse. These mice were bred with floxed GCS-mice (Jennemann et al. 2005) in order to stop glucosylceramide *de novo* biosynthesis.

3.4.1. Analysis of the phenotype of GCS-deficient mice

KO mice were undistinguishable from controls at birth. The bodyweight increased for both, GCS-deficient and control animals until P2 (2 days after birth) (see Fig. 20). Starting from P3 (3 days after birth) the weight in GCS-KO mice stagnated and during further development decreased. In comparison to their respective control littermates, the bodyweight at P4 (4 days after birth) was only half of that of controls (Jennemann et al. 2006).

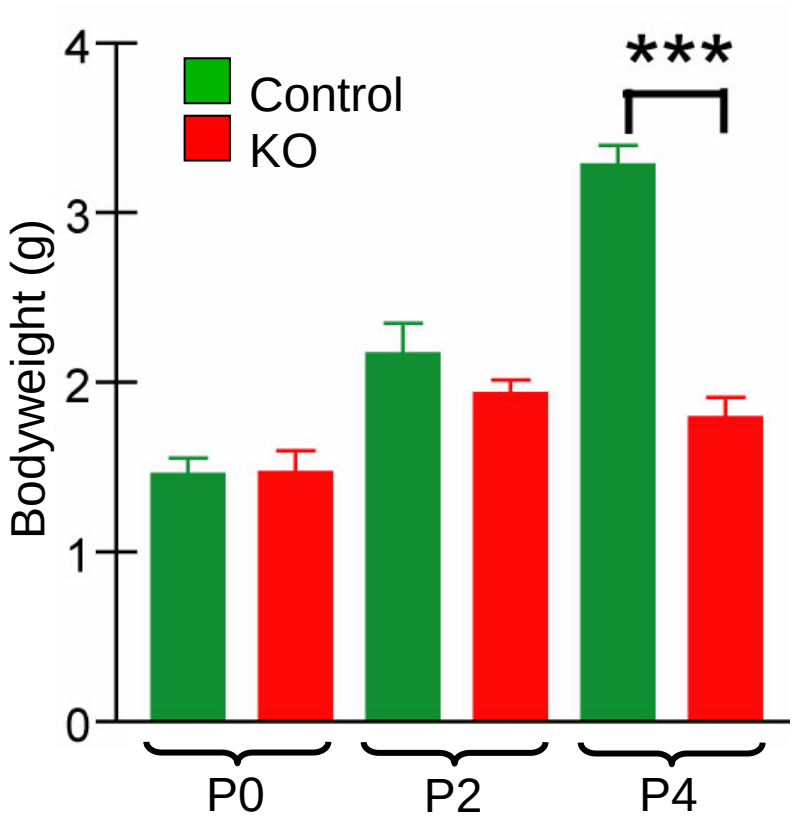

Fig. 20. Evolution of the bodyweight of GCS-deficient mice relative to wildtype littermates. The bodyweight was measured at birth (P0), and at days 2 (P2) and 4 (P4). (Jennemann et al. 2006)

KO-mice showed no skin abnormalities at birth (Fig. 21A and 21C, (Jennemann et al. 2006)). This changed by days 3 to 4, as desquamation began to appear at the joints, around the mouth, and on parts of the skin. This desquamation expanded in the subsequent days to cover

large areas of the body (Fig 21B and D). Hematoxylin/eosin (HE)-staining of different skin regions demonstrated that skin desquamation exclusively involved the *stratum corneum* (Fig. 21D2; compared to 21D1 intact skin and 21D3 skin section as indicated without *stratum corneum*). When skin was incubated with Lucifer yellow solution, diffusion of the fluorescent dye in control animals was restricted to the outer sheaths of the corneal layer (Fig. 21B1'). Regions of GCS-deficient animals without detachment of the corneal layer had an intact skin barrier (Fig. 21D1'). However, facilitated Lucifer Yellow diffusion was observed in areas with intense skin peeling. In addition, a restricted hair development was observed at P4 (Fig. 21D, GCS-deficient *vs.* Fig. 21B, control).

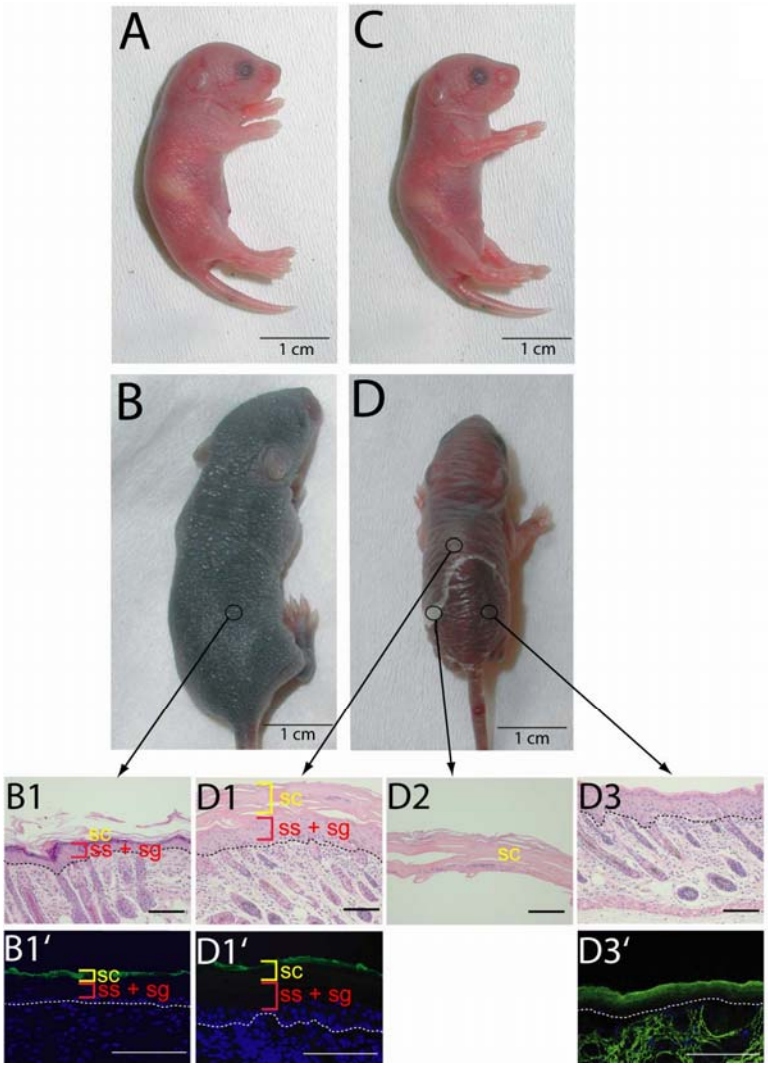

Fig. 21. Analysis of the phenotype of GCS-deficient mice. A-D, Photographs of mutant mice (C and D) and control littermates (A and B) at P0 (newborn, A and C) and at P4 (B and D). B1-D3, hematoxylin/eosin (HE)-staining of skin sections from control (B1) and GCS-KO mice (D1-D3). Bars: 100µm. D1, reflects a region in which the epidermal *stratum granulosum* (sg)-*stratum corneum* (sc) junction is not yet dissociated fully. Peeled skin consists solely of cells of the *stratum corneum* layer (sc, D2). D3, Cell layers under the peeled skin shows a complete loss of the *stratum corneum*. Only *stratum granulosum* and the *stratum spinosum* (sg and ss) layers could be seen. B1'- D3', Lucifer yellow diffusion in wildtype (B1') and in regions of GCS-deficient animals without detachment of the *stratum corneum* (D1'). A destruction of the skin permeability barrier was observed indicated by Lucifer yellow diffusion throughout the whole skin in regions in which the *stratum corneum* layer was lost (D3'). (Jennemann et al. 2006)

A detailed microscopical analysis showed that the morphology of newborn wildtype and KO mice does not differ (data not shown). This changed as older GCS-deficient mice started showing an absence of granules in the *stratum granulosum* (P4). These mice also show a thicker epidermis and stratum corneum. There was also a significant increase in proliferation in the *stratum basale*, shown by immunohistochemistry using the Ki67 antibody. GCS-deficient mice also show reduced cell viability. TUNEL assays showed increased apoptosis at the *stratum granulosum-stratum corneum* interphase, compared to wildtype. It is interesting to note an increase in the number of monocytes, macrophages, and dendritic cells in the dermis of P5 GCS-deficient mice, compared to controls of same age.

The measurement of transepidermal water loss (TEWL) using a Tewameter® TM 300 at sites of the skin were extreme desquamation occurred (P4), showed TEWL values around 20 g $H_2O/h/m^2$. This indicates a loss of function of the water permeability barrier. As result of water loss all mutant animals died within 5 days of birth (Jennemann et al. 2006).

3.4.2. Lipid analysis of GCS-deficient mice

The lipid analyses were carried out on skin of mice aged 0, 2 and 4 days after birth. From these, complete skin was taken and processed as described in materials and methods. The extracted free lipids of epidermis and SC, as well as covalently bound lipids of the SC were analyzed with HPTLC. The amounts of ceramides were quantified using densitometry (Jennemann et al. 2006).

Fig 22 shows that the ceramide profile of KO mice does not differ from that of wildtype except at the stage P4, where Cer(EOS) as well as Cer(NP) is less abundant than in the previous 2 stages. Interestingly, there is a higher amount of Cer(EOS) in P4 KO than in P4 wildtype. nanoESI-MS/MS Mass spectrometry analysis of lipid extracts of epidermis of GCS-deficient mice showed that non esterified ω-hydroxylated ceramides accumulated significantly compared to wildtype.

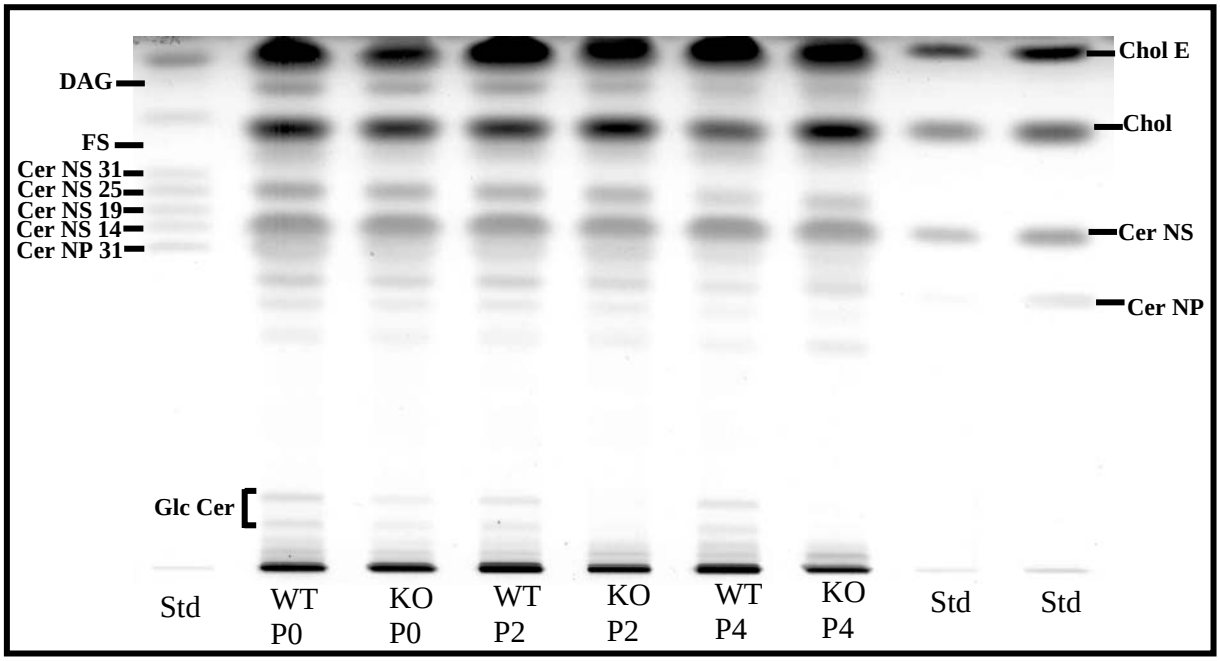

Fig. 22. Free epidermis lipids. Separation was performed on HPTLC plates using Chloroform/Methanol/Acetic Acid (190:9:1, v/v/v) and developed 2 times for ceramides. The plate was air-dried, sprayed with 8% (wt/vol) H_3PO_4 containing 10% (w/vol) $CuSO_4$ charred at 180°C for 10 min. WT: wildtype. KO: Knock out. P0, 2, and 4: 0, 2, and 4 days, respectively, after birth. Abrv: Chol: Cholesterol; Chol-E: Cholesterol Ester. (Jennemann et al. 2006)

The separation of GlcCers shows a progressive reduction of GlcCers in KO mice compared to wildtype (Fig. 22). This decline starts at stage P2, and at stage P4, the rest amount of GlcCers is 50%. Furthermore, the amount of the more hydrophilic sphingomyelin (Fig. 23, SM band A) decreased in deficient mice, compared to wildtype. This is confirmed by nanoESI-MS/MS mass spectrometry which shows a 50% decrease in C16 SM(AS) (age P4).

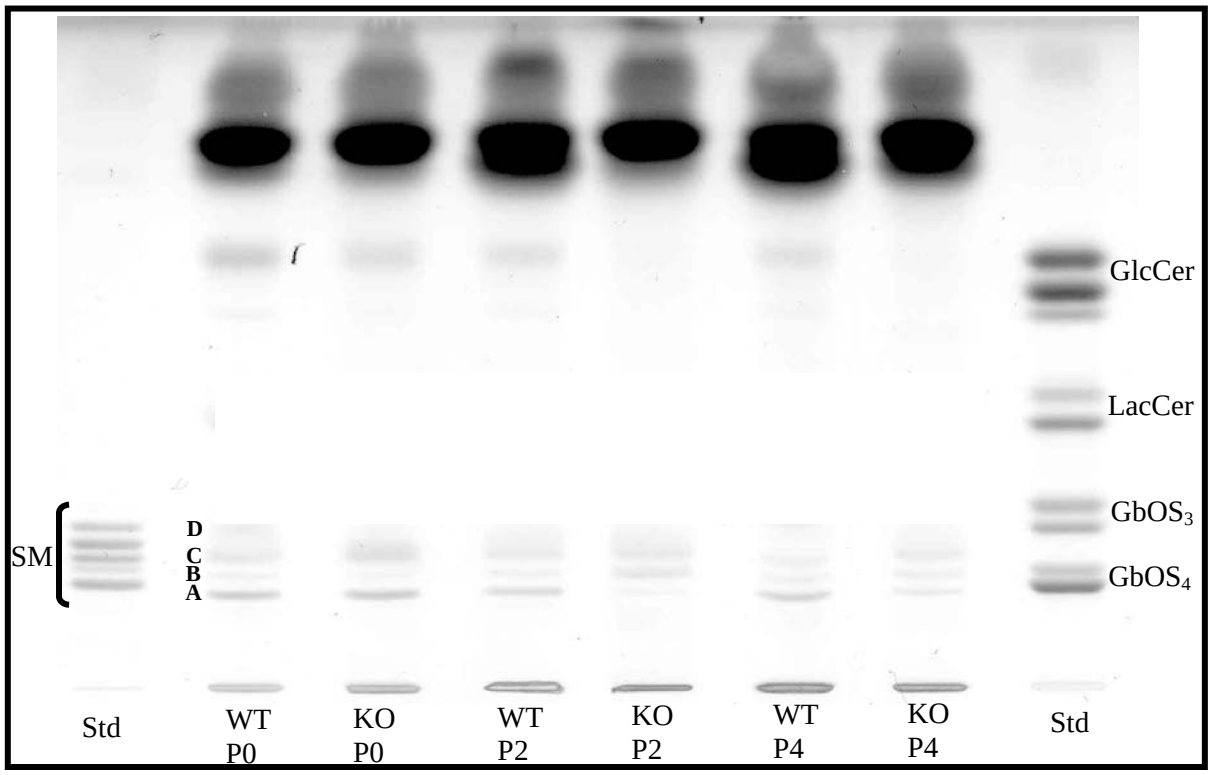

Fig. 23. Free epidermis lipids. Separation was performed on HPTLC plates using Chloroform/Methanol/Water (70:30:5, v/v/v) and developed 1 time for glucosylceramides. The plate was air-dried, sprayed with 8% (wt/vol.) H_3PO_4 containing 10% (w/vol) $CuSO_4$ charred at 180°C for 10 min. WT: wildtype. KO: Knock out. P0, 2, and 4: 0, 2, and 4 days, respectively, after birth (Jennemann et al. 2006).

The covalently bound lipids were extracted from the *stratum corneum* by mild alkaline hydrolysis after ensuring that free lipids were no longer present in the samples. The lipids were then separated on HPTLC plates and quantified by densitometry. The results show that KO mice have lost over 60% of their ω-hydroxylated Cer(OS) and 76% of Cer(OH) (Fig. 24). The ω-hydroxylated fatty acids are also reduced by 50%. On the other hand, GCS-deficient mice show a 6-folde increase in Cer(NP), as well as a less dramatic (10%) increase in free fatty acids. Interestingly, KO mice show the appearance of an unknown band under that of Cer(OH) (Fig. 24 question mark). The overall picture shows slight alterations in the profiles of covalently bound lipids to be found in wildtype and GCS-deficient mice.

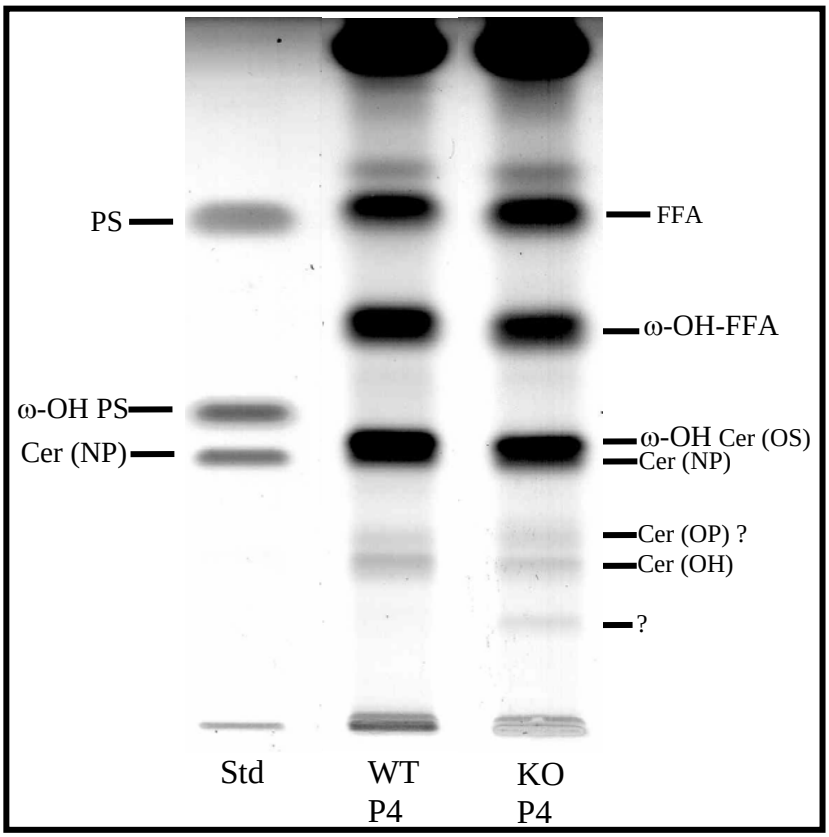

Fig. 24. Covalently bound lipids od the *stratum corneum*. Separation was performed on HPTLC plates using Chloroform/Methanol/Acetic Acid (190:9:1, v/v/v) and developed 2 times for ceramides. The plate was air-dried, sprayed with 8% (wt/vol) H_3PO_4 containing 10% (w/vol) $CuSO_4$ charred at 180°C for 10 min. WT: wildtype. KO: Knock out. P4: 4 days after birth. PS: palmitic acid. ω-OH-PS: ω-hydroxylated palmitic acid. FFA: Free fatty acids (Jennemann et al. 2006).

4. Discussion

4. Discussion

Keratinocytes are difficult to study in the laboratory due to many reasons. The principle reason is that the epidermis, much like the brain, is composed of many different types of cells, or of cells of the same type which are in different stages of their cell cycle, either proliferating, or going through the different stages of terminal differentiation. In our laboratory, it was possible to define and establish a cell culture system which enables keratinocytes to behave much like in native epidermis. We are able thus to induce keratinocytes to proliferate (low calcium medium), or to differentiate (calcium shift and high calcium medium). We were also able to define a cell culture system based on the serum free medium MCDB-153, with addition of calcium (1.1 mM) and linoleic acid (10 μM). This medium enabled keratinocytes to differentiate and synthesize the same pattern of lipids as native epidermis. Through the induction of differentiation by the calcium shift, we have cells which are nearly synchronous in their development. Thus, they undergo the different stages of differentiation, starting from the *stratum basale* before the calcium shift, through the *stratum spinosum* to the *stratum granulosum*. Our cell cultures are unable to reach the *stratum corneum* stage. This is probably due to the fact that air exposure and intact desmosomes are needed for this to occur. Colleagues in the laboratory have previously analysed the lipid profiles of these cells under the different culture conditions (Döring 1999; Breiden 2003). The next step in characterizing this culture medium was to analyse the mRNA expression profile of cells in low calcium medium. To this end, we chose proteins which are known to be markers of proliferation (Keratin 14) and differentiation (keratin 10, profilaggrin), as well as enzymes of the ceramide metabolism. In order to accomplish this, we used real time quantitative PCR and established for each measured protein a Primer and Probe set, as well as optimized the reaction conditions for each set of primers.

4.1. mRNA expression pattern of keratinocytes in different culture media

Linoleic acid is important for the formation of the lipid barrier as it is attached to ω-hydroxylated ceramides in ω position, and thus is part of the long chain ceramides which are essential for barrier formation. The addition of Linoleic acid (10 μM as a BSA complex) to the culture medium at the calcium shift enhanced the expression of all measured markers, but in

varying degrees. The highest increase is measured for acid sphingomyelinase and acid ceramidase, two hydrolytic enzymes regulating the amount of ceramides present in the cell.

The addition of Vitamin C to 3D-keratinocyte cultures induces an increase in the overall ceramide level as well as a change in the ceramide profile synthesized by the cells toward that of native epidermis (Ponec et al. 1997). The addition of 50 µg/ml of vitamin C to submerged keratinocyte cultures resulted in an increase in α- and ω-hydroxylated sphingolipid synthesis, an increase in covalently bound lipids, and an increase in lamellar body production and secretion (Uchida et al. 2001). The analysis of the expression pattern of ceramides revealed an increase in the synthesis of Cer(AS)/(NH) as well as Cer(AP)/(AH) (factor 1.4), and Cer(NP) (factor 1.7). The Cer(NS) fraction increased by a factor of 1.2, whereas Cer(EOS) remained unchanged. The synthesis of covalently bound lipids also changed due to vitamin C. The ω-hydroxylated fatty acids increase 1.2 fold. The non-hydroxylated fatty acid content increased 2.6 fold, and that of Cer(OS) increased 1.2 fold (Breiden 2003). These effects were not mirrored in the mRNA expression levels of the measured markers, suggesting that vitamin C acts not as an expression regulator, but rather as a cofactor of enzyme activity.

4.2. The mRNA expression profile of differentiating keratinocytes

The formation of the water permeability barrier in the stratum corneum is a crucial and complex step toward the constitution of a fully functional skin. The importance of ceramides for the function of the water barrier is highlighted by the Collodion babies phenotype, observed in the most severe form of Gaucher´s disease, and their mouse models, where a complete loss of β-glucocerebrosidase activity results in high rates of transepidermal water loss leading to death a few days after birth (Lui et al. 1988; Doering et al. 1999b). In order to better understand the formation of ceramides during the differentiation of keratinocytes, we analyzed the mRNA levels of proteins involved in ceramide metabolism of cultured human foreskin keratinocytes during differentiation, as initiated by the addition of calcium ions (1.1 mM) and linoleic acid (10 µM) to the culture medium.

The mRNAs of β-glucocerebrosidase and glucosylceramide synthase were upregulated more than 30-fold, which is consistent with the increased activities of these enzymes and with

their proposed role in the formation of ceramides for the water permeability barrier. Complex glucosylceramides are considered to be the precursors of the free and protein-bound ceramides, which are generated by the combined action of β- glucocerebrosidase and the protein Sap C (Doering et al. 1999a).

The expression level of the key enzyme of de novo sphingolipid biosynthesis, serine palmitoyltransferase, was only increased 2- to 3-fold during calcium shift. Also *de novo* ceramide biosynthesis increased transiently only up to 2-fold within the first 24 hours after calcium shift. This observation suggests that the synthesis of ceramides used in the water barrier formation is predominantly achieved by turnover of sphingolipids, especially by the breakdown of pre-existing lipids, such as sphingomyelin and glycosphingolipids, by lysosomal proteins and not only by *de novo* biosynthesis. This view is supported by the up to 5-fold increased expression of acid sphingomyelinase and 30-fold increase of β- glucocerebrosidase after the calcium shift, whereas the level of acid ceramidase mRNA increased only slightly. The up to 16-fold increase of prosaposin mRNA also underlines the important role of Saps in sphingolipid metabolism of the skin. Sap-C is needed for the catabolism of free and protein-bound glucosylceramides (Doering et al. 1999b) to generate the respective ceramides. This is in accordance with our observation that the maximal increase in pSap mRNA level occurs toward 8 days of culture. Thus, the expression pattern of these enzymes render of picture of keratinocytes which, after the calcium shift, change the expression pattern of the enzymes involved in the ceramide metabolism toward increase synthesis of so called probarrier lipids. These changes are also time-dependent, indicating that cells, as they differentiate through the different stages of terminal differentiation, modulate their expression profiles in order to create a lipid barrier. The increase of glucosylceramide synthase and β-glucocerebrosidase mRNA after 8 days suggests that the cells are at the stage of stratum granulosum at this time, where probarrier ceramides are increasingly transformed into glucosylceramides and packed in lamellar bodies to be secreted in the intercellular space of the stratum corneum, together with β-glucocerebrosidase. Since this enzyme needs Sap-C to be active, it is no surprise that the level of prosaposin, the precursor of Sap-C, increase after 8 days, in parallel to β-glucocerebrosidase. The mRNA measurements also show an early (after 6 hours) increase in keratin 10 (30 fold compared to time point 0 hours). This confirms the status of keratin 10 as an early marker of keratinocyte differentiation. Profilaggrin, a protein necessary for the constitution of barrier proteins is strongly increased (50 fold increase),

but toward the end of differentiation (8 days). This is also consistent with its described role as an agent of keratin filament packing.

All these mRNA measurements indicate that, and are consistent with, keratinocytes preparing to fulfil their role in the establishment of the lipid permeability barrier of the stratum corneum through the synthesis of the necessary proteins and lipids, and the enzymes necessary for their processing.

4.3. Effect of NO on fibroblasts and on keratinocyte differentiation

Nitric oxide (NO) has received increasing attention because of the very interesting, varying and sometimes confusing effects it has on different cell types, and sometimes on the same cell type at different stages or in different concentrations. It is generally considered to be an inducer of apoptosis in many cell types (Sandau and Brune 1996; Suschek et al. 1999), even though NO protects other cell types from apoptosis (Dimmeler et al. 1997; Li et al. 1999).

Keratinocytes express NOS genes (Shimizu et al. 1997), and it has been shown that NO stimulates keratinocyte proliferation (Krischel et al. 1998) and inhibits cornified envelope formation (Rossi et al. 2000). Therefore, we tested the effects of NO on differentiating keratinocytes. Whereas the addition of a NO donor to the culture medium apparently did not affect the morphology of differentiating epidermal keratinocytes, it generated dramatic effects in dermal fibroblasts as they underwent apoptosis within 6 days. On the other hand, inhibition of endogenous NO production by addition of the NOS inhibitor L-NAME had no effect on cultured fibroblasts but in differentiating keratinocytes unexpectedly induced a process which led to apoptosis after 5 days.

The apoptotic process initiated in keratinocytes by the addition of L-NAME was accompanied by a drastic decrease of the mRNA levels of all differentiation markers and proteins of ceramide metabolism. At the lipid level, however, biosynthetic ceramide labelling increased transiently to twice that of untreated cells after 24 hours and then dropped to rather low levels. The transient increase in ceramide formation, which may have triggered an apoptotic signal, could be caused at least in part by the early drop of GCS mRNA expression and the coinciding

decrease of glucosylceramide and sphingomyelin formation (Macheleidt et al. 2002). On the other hand, the addition of the NO donor SNAP (0.2 mM) to the differentiating keratinocytes caused no apoptosis but triggered an increased proliferation. The concomitant decrease of the differentiation marker keratin 10 mRNA and increase of the basal marker keratin 14 mRNA suggest that keratinocytes remained in a proliferative state even in the presence of high extracellular calcium concentrations. This assumption is supported by thymidine incorporation studies we conducted in keratinocyte cultures (El Massoudi, 1999). The addition of 0.05 mM SNAP to the culture medium after calcium shift resulted in an increase of the proliferation rate by 60%. A similar proliferative response was also induced by SNAP (range 0.05 to 0.25 mM) in wound healing processes (Albina et al. 1990; Krischel et al. 1998).

Skin wound healing is accompanied by an induction of iNOS (Frank et al. 1999; Lee et al. 2001) and elevated levels of nitrites and nitrates in wound fluids (Uchida et al. 2000). A role for sphingosine-1-phosphate has also been described in this process. Studies suggest that sphingosine-1-phosphate is synthesized in platelets in which they are stored. In the event of a wound, sphingosine-1-phosphate is released and triggers keratinocyte proliferation (Lee et al. 2000). However, 1α,25-dihydroxyvitamin D3 induces in keratinocytes an increase in sphingosine-1-phosphate, at the same time inhibiting proliferation (Manggau et al. 2001). This is in contrast to the suggested role of S1P in wound healing as inhibition of cell growth of keratinocytes prevents efficient closure of the wound. But it has to be considered that a variety of mediators are released from platelets at the wounded site. Growth factors such as epidermal growth factor, platelet-derived growth factor, TGF-β, and fibroblast growth factor are stored or formed in thrombocytes, many of them affect keratinocyte cell function, allowing a complex interplay. In astroglial cells, the biosynthesis of tetrahydrobiopterin (BH4), the coenzyme required for NO synthesis and hydroxylation of tyrosine and tryptophan, is stimulated by various proinflammatory cytokines, including TNF-α. These induce expression of GTP-cyclohydrolase, the rate-limiting enzyme in the *de novo* pathway for BH4 biosynthesis. TNF-α stimulates iNOS, which requires BH4 for its activity, and GTP-cyclohydrolase expression by divergent pathways (Vann et al. 2000). Whereas TNF-α stimulated iNOS expression via a ceramide-dependent pathway, it also regulated GTP cyclohydrolase expression independently of ceramide generation. The sphingolipid metabolite sphingosine-1-phosphate, but not ceramide, potentiated GTP-cyclohydrolase mRNA expression induced by TNF-α. Conversely, TNF-α- induced Sphingosine

kinase activation and generation of sphingosine-1-phosphate did not modulate expression of iNOS. Thus TNF-α induces the coordinate expression of iNOS and GTP cyclohydrolase via ceramide- and sphingosine-1-phosphate -dependent pathways, respectively (Vann et al. 2000).

In our keratinocyte cultures this state, as triggered by the addition of the NO donor SNAP, was accompanied by a marked reduction of the mRNA levels of most enzymes involved in ceramide metabolism as well as of prosaposin and, in contrast to apoptotic cells in the presence of the NOS inhibitor L-NAME, also by a transient drop in the de novo formation of ceramides, but with the persistence of the proliferation marker keratin 14. It is conceivable that the wound healing process involves differentiating keratinocytes to revert to a proliferating basal state in order to close the existing wound gap. After this is achieved, newly synthesized cells can then differentiate to reconstitute the epidermis and the integrity of the permeability barrier. Our results together with the afore-mentioned studies show that nitric oxide is in this context one of the major elements influencing the wound healing process by inhibiting differentiation and stimulating proliferation at its place. The molecular mechanisms for the inhibition of terminal differentiation by NO remain to be elucidated. However, it is likely that this effect is based on the inactivation of transcription factors such as AP-1 by Snitrosylation of cysteine residues (Tabuchi et al. 1994; Rossi et al. 2000). Akool et al. (Akool el et al. 2003) have also shown a mechanism of action through which NO reduced the expression of the mRNA-stabilizing factor HuR which led to an increased decay of the matrix metalloproteinase 9 mRNA, which is a response element to inflammatory cytokines such as interleukin-1 beta in rat mesangial cells. The mechanism was also shown to be cGMP dependent. NO diffuses through the plasma membrane and activates a cytosolic guanylate cyclase. This increases the concentration in cGMP (Siragy et al. 1992) and leads to the phosphorylation of target proteins through cGMP-dependent protein kinase G. This reduces the intracellular calcium concentration (Waldman and Murad 1988).

We studied the effect of cGMP on sphingolipid metabolism. In keratinocyte cultures, in which cells were incubated in the presence of cell-permeable analogs of cGMP (8-bromo-cGMP or dibutyryl-cGMP in concentrations ranging from 50 to 250 μM), the amount of newly synthesized ceramide, as well as the ceramide patterns, remained unchanged (data not shown). The simultaneous addition of L-NAME (10 mM) and 8-bromo-cGMP (0.5 mM) to differentiating keratinocytes and subsequent incubation for 24 hours had no effect on the amounts of ceramides

and glucosylceramides synthesized, compared to controls cultivated with only L-NAME. These findings indicate that the influence of NO on the sphingolipid metabolism is not dependent on cGMP. However, the possibility cannot be discarded that nitric oxide acts through other, non cGMP-dependent, and keratinocyte-specific pathways in order to direct the cell cycle, in the event of cell stress such as wounds, toward either proliferation or differentiation. This possibility must be considered and studied in depth. An implication of this possibility may well be that NO also plays a role in either initiating or inhibiting some hyperproliferative skin diseases such as psoriasis. It is also possible that in such cases, the pathological phenotype is allowed to progress due to a failed regulation of the proliferation process by central control points, one of which could presumably be NO. The addition of L-NAME to differentiating keratinocytes induced apoptosis after about 5 days of the calcium shift. The apoptotic signal resulted in a marked reduction in expression levels of many enzymes of the sphingolipid pathway such as serine palmitoyltransferase, acid ceramidase, β-glucocerebrosidase, and acid sphingomyelinase, with others such as glucosylceramide synthase tending practically toward zero. This may suggest that the reduced levels of gene products are more the result of the onset of apoptosis, rather than a direct effect of the NOS inhibitor. On the other hand, we cannot discount the possibility that the lysosomal proteins are quite stable against proteolysis and remain active - e.g. in generating ceramides from the precursor glucosylceramides and sphingomyelin - even after their mRNA levels already dropped to negligible values. The increased ceramide levels, particularly those of short chain ceramides, and not the vary long chain ceramides involved in permeability barrier formation, may also be involved in the onset of apoptosis. To investigate this point, we did metabolic incorporation studies using two different inhibitors (conduritol-B-epoxid and fumonisin B1) blocking ceramide biosynthesis through glucosylceramide catabolism (CBE) or synthesis from sphingosin or *de novo* (fumonisin B1). We were able to show that L-NAME induces an increase in ceramide through enhancing the activity of acid sphingomyelinase. Since the synthesis of ceramides through sphingomyelin turn-over takes only one step, it is logical that cells, if they are inducing apoptosis through the action of ceramide, are able to generate very quickly large amounts of ceramides through this pathway. These results indicate that NO plays a role not only in the regulation of ceramide metabolism but also seems to have a central role in controlling the terminal differentiation of keratinocytes.

4. Discussion

4.4. Study of the glucosylceramide KO mouse

The importance of the water permeability barrier to land dwelling organisms was shown through the dramatic effect of complete loss of function of β-glucocerebrosidase resulting in the collodion baby phenotype. Through the analysis of this and a Sap-C deficient mouse (Doering et al. 1999a), our laboratory constructed a model for water permeability barrier establishment. In this model, probarrier lipids such as ceramides are synthesised in the endoplasmatic reticulum. They are then transferred to the Golgi where they are glucosylated through the action of the glucosylceramide synthase. In the case of Cer(EOS), the very long chain ω-hydroxylated linoleic acid esterified ceramides, one of the most important species of ceramides, it is unknown whether the linoleic acid esterified at the ω position is added in the endoplasmatic reticulum (before) or in the Golgi (after) glucosylation. These glucosylated probarrier lipids are then packed in the lamellar bodies in the *stratum granulosum*, and are extruded in the extracellular space in the *stratum corneum*. These ceramides are then attached to the proteins of the cornified envelope by ester bounds mediated through transglutaminases and the glucose rest is then cleaved by the β-glucocerebrosidase. The absence of β-glucocerebrosidase or of Sap-C (an important helper of β-glucocerebrosidase) resulted in an impaired permeability barrier leading to the collodion phenotype. We sought to investigate the effect of glucosylceramide synthase, another enzyme heavily involved in the genesis of the barrier. The question we were trying to answer is whether ceramides need to be in a glucosylated form in order to be attached to the cornified envelope.

The systemic disruption of glucosylceramide synthase is lethal during embryogenesis. In order to study the effect of glucosylceramide synthesis knockout in the nervous system, a tissue specific knockout, driven by the promoter for nestin, a nervous system-specific protein, resulted in death approximately 3 weeks after birth (Jennemann et al. 2005). The same system was used by the group of Gröne (DKFZ, Heidelberg) to generate a constitutive skin-specific KO mouse. The K14 promoted Cre-recombinase mice used in this study for the specific elimination of the glucosylceramide synthase in the epidermis showed a strong but mosaic-like Cre-mediated recombination pattern at E15 (Huelsken et al. 2001). However, it could be shown that the Cre-recombinase expression was ubiquitously distributed in the complete basal cell layer of the epidermis and in hair follicles after birth. These GCS-deficient mice weight only 57% of wildtype

mice 4 days after birth. They have a transepidermal water loss 6 times higher than wildtype, and die 5 days after birth.

The analysis of the lipid patterns of the skin of these KO mice showed that the residual GlcCer amount drops to about 50% of wildtype 4 days after birth. The residual amounts of GlcCers observed in P4 KO-mice can be explained by the glucosylceramides synthesized before activation of the Cre-recombinase, together with an initially slow degradation of glycosphingolipids. This changed after birth, as the expression of β-glucocerebrosidase increased, effectively reducing the amount of GlcCer.

The ceramide patterns of wildtype and KO mice remained unchanged, except for Cer(EOS) which was reduced in KO mice at stage P4. Cer(EOS) has a linoleic acid in ester linkage to the ω-hydoxy-group of the fatty acid. This ceramide species is thought to act as an anchor of the lipid lamellae to the protein matrix to which Cer(EOS) is covalently bound. This is possibly a factor contributing to the flexibility of the *stratum corneum*, without which it is liable to crack under mechanical stress. This could explain the appearance of desquamation at the joints and around the mouth first, which are regions of great mechanical stress for the skin, spreading afterwards to other regions.

As mentioned in the introduction, ceramides are synthesized in the endoplasmatic reticulum. They are then transferred to the Golgi, and glucosylated. It is not known whether, in the case of Cer(EOS), the linoleic acid ester-linked at the ω position is added before (in the endoplasmatic reticulum) or after (Golgi) glucosylation. The accumulation in the skin of GCS-deficient mice of the ceramide species with non-esterified ω-hydroxylated long chain fatty acids and the simultaneous reduction of Cer(EOS) is an interesting finding with regard to the topology of glucosylceramide biosynthesis as it suggests that ceramides are transported to the Golgi, glucosylated, and then esterified to linoleic acid, in order to establish the Cer(EOS) species. The blocking of the glucosylation step could thus mean that Cer(EOS) could not be synthesized in sufficient amounts to act as an anchor to the *stratum corneum*. This may also explained the detached stratum corneum seen in GCS-deficient mice.

4. Discussion

The survival of GCS-deficient mice to 5 days after birth indicates that the skin has a mechanism to compensate for the reduced amounts of GlcCers generated by biosynthesis. This compensatory pathway seems to use sphingomyelin to replace GlcCer. We measured a 2 fold increase in long chain sphingomyelins SM(OS) and SM(EOS) in KO mice. These sphingomyelin species could then be attached to the stratum corneum, then have their phosphocholin headgroups cleaved. This would result in the formation of a rudimentary water barrier which would be good enough to prevent immediate death of KO mice. It is possible that this process is far slower than the pathway through GlcCer, either in the synthesis of the long chain SM(EOS), or the cleavage of the phosphocholin headgroup, thus preventing mice from regenerating the water barrier as fast as natural desquamation occurs, thus resulting in a thinner *stratum corneum*, which is also more easily breakable. Another possibility is the destabilizing effect of the phosphatidylcholine headgroup, due to its higher polarity compared to glucose. This would lead to a disturbed structure of the lamellae which are formed of hydrophobic lipids.

Thus, the desquamation phenotype observed in these mice is probably a result of both a disturbance of the lamellar arrangement of the lipid layers, together with a faulty anchoring of these layers to the protein matrix. This would lead to a detached stratum corneum as well as a porous lipid barrier. The result would be an increased transepidermal water loss, measured as 6 times higher than wildtype mice, and subsequently dehydration and death.

The skin of GCS-deficient mice also showed another interesting phenotype which is an increased proliferation of the stratum basale. This could be the result of an inflammation response mechanism triggered by the defective *stratum corneum*, through which keratinocytes of the basal layer were induced into increasing their proliferation rates. This led to a hypertrophied *stratum basale*. However, there was no significant increase in the thickness of the differentiation keratinocytes in the *stratum granulosum*. It is known that an increase in the ceramide content of cells results in a signalling cascade which leads to a reduction of proliferation as well as an induction of differentiation and/or apoptosis. Since the silencing of the GCS gene lead to an increase in proliferation, this means that no ceramides were involved in this process. Accordingly, the analysis of the lipid patterns of GCS-deficient mice showed that the blocking of this pathway lead to an increase in ceramide Cer(OS) which plays a role in the lipid barrier genesis. The content in other ceramides did not change. On the other hand, it is possible that

ceramides were degraded to sphingosine which, after phosphorylation to sphingosine-1-phosphate, started a signalling cascade leading to a proliferation signal in the basal layer. The ceramides which were used for this pathway may have been compensated through sphingomyelin degradation, as short chain sphingomyelin NS and AS was strongly reduced in GCS-deficient mice, whereas Cer(NS/AS) remained unchanged.

In conclusion, the tissue specific constitutive silencing of the glucosylceramide synthase gene in mice led to a disturbed lipid barrier. The absence of glucosylation of Cer(OS) resulted in a reduced availability of probarrier lipids with the linoleic acid in ω position necessary for barrier formation. This deficit was partially compensated through a sphingomyelin pathway. However, sphingomyelin with its phosphocholin headgroup seems not to be as well adapted for this task, as GCS-deficient mice suffered from a detached and weak stratum corneum which lead to death 5 days after birth due to a vary high transepidermal water loss. These mice also displayed a skin with a hyperproliferating stratum basale, without reduction in intracellular ceramide levels, but rather through a reduction in sphingomyelin NS/AS generating corresponding ceramides which were then degraded to sphingosine.

5. Materials and Methods

5.1. Cells and Reagents

Human foreskin keratinocytes were kindly provided by Prof Dr. Kaiser (Department of Dermatology, University of Bonn, Germany). MCDB-153 medium and serine deficient MCDB-153 medium for keratinocyte cell culture was obtained from Biochrom KG seromed® (Berlin, Germany). DMEM medium and primers were purchased from Gibco BRL-Life Technologies (Paisley, Scotland). SNAP and L-NAME were obtained from Alexis-Biochemicals (Grünberg, Germany). Fluorogenic probes, MuLV RT, and AmpliTaq Gold® were purchased from Applied Biosystems (Weiterstadt, Germany). Uracil-N-glycosylase and RNase free-DNase I where obtained from Roche diagnostics (Indianapolis, IN) and RNasin® from Promega (Heidelberg, Germany). The Purescript® RNA isolation kit was obtained from Gentra Systems (Minneapolis, MN). Pyridoxal phosphate, palmitoyl-CoA, 4-methylumbelliferyl-β-D-glucopyranoside, 4-methylumbelliferone, Mouse monoclonal Anti-cytokeratin 10 antibody clone 8.60 C, fluorescence coloring "H 33258" (Hoechst No. 33258), and deoxyribonucleic acid sodium salt from salmon testis were obtained from Sigma (Deisenhofen, Germany). Methanol, chloroform, LiChroprep® RP-18, and TLC plates (20 x 20 cm, Silica Gel 60) were obtained form Merck *eurolab* (Darmstadt, Germany) and trypsin was from Promo Cell (Heidelberg, Germany). Labeled L-[3-^{14}C]-serine (54 Ci/mol) was from Amersham Biosciences Europe GmbH (Freiburg, Germany). Liquid scintillation counting was carried out with Ultima-Gold - scintillation cocktail (Packard Instruments B.V., Groningen, The Netherlands). The ApoAlert® Caspase-3 colorimetric assay kit was obtained from Clontech laboratories (Palo Alto, CA). Supersignal West Dura Mouse IgG detection kit was obtained from Perbio science (Bonn, Germany).

5.2. Cell Culture

Human foreskin keratinocytes were cultivated according to a modification of the Rheinwald and Green method (Rheinwald and Green 1975) in MCDB-153 medium in a vapor saturated atmosphere with 5% CO_2. The medium (containing 210.7 mg/l arginine·HCl) was supplemented with insulin (5 µg/ml), hydrocortisone (0.4 µg/ml), ethanolamine (0.1 mM), phosphoethanolamine (0.1 mM), epidermal growth factor (0.1 ng/ml), bovine pituitary extract (100µg/ml), L-histidine (0.24 mM), L-isoleucine (0.75 mM), L-phenylalanine (90 µM), L-tryptophan (45 µM), L-tyrosine (60 µM), and N-acetyl-L-alanyl-L-glutamine (4.8 mM). Cells were grown in low calcium (0.1 mM) MCDB-153 medium to confluency.

Differentiation was initiated through a calcium shift to 1.1 mM and addition of linoleic acid/BSA (10 μM) in MCDB-153 medium. Human fibroblasts were cultivated in DMEM medium (10% fetal calf serum). SNAP was solubilized in methanol and L-NAME in MCDB-153 or DMEM medium. Each was then added to the cell cultures together with the medium change, which took place every 24 hours. All cell culture experiments were conducted at least in duplicates.

5.3. Ceramide Labeling

Human epidermal foreskin keratinocytes were cultivated as described above. 6 h before harvesting, the medium was changed to the corresponding serine-deficient medium and L-[3-^{14}C]-serine was added (1 μCi/ml medium). The cells were harvested at indicated time points after calcium shift (Fig. 1) with 0.25 % trypsin in PBS for 15 min at 37 °C. The incorporated radioactivity was quantified with a liquid scintillation counter (Packard Instruments Company, *Tri-Carb 1900 CA*, Connecticut, USA), and visualized after TLC-separation by phospho-imager analysis (FUJIX BAS 1000, Fuji Photo Film Co., Ltd., Tokyo, Japan) in order to quantify the relative distribution of the radioactivity on the TLC plate. The phospho-imager measures the degree of blackening of the radiography film expressed in photo stimulated luminescence (PSL) units.

5.4. Preparation of Epidermis

Whole skin was removed at autopsy from newborn mice. Epidermis was separated from dermis after floating the skin on Dispase (Boehringer Mannheim, grade II) diluted 1:1 in Hanks' buffer at 4 °C overnight.

5.5. Lipid extraction and analysis

Tissue samples were homogenized, lyophilized, and weighed. Epidermal lipids were extracted for 24 h at 37 °C in each of three solvent mixtures (chloroform/methanol/water 1:2:0.5 (v/v/v); chloroform/methanol 1:1 (v/v), and chloroform/methanol 2:1 (v/v). Total lipid extracts were applied to thin-layer Silica Gel 60 plates (Merck Darmstadt, Germany). All plates were washed with chloroform/methanol (1:1, v/v) before sample application. For separation of glucosylceramides, the chromatograms were developed once with chloroform/methanol/water (70:30:5, v/v/v). Ceramides were resolved twice using chloroform/methanol/acetic acid (190:9:1, v/v/v) as developing solvent. For quantitative analytical TLC determination, increasing amounts of standard lipids (*N*-stearoyl-sphingosine

(kind gift of Beiersdorf AG, Hamburg, Germany), sphingomyelin (Sigma), and glucosylceramide) were used. After development, plates were air-dried, sprayed with 8% (w/v) H_3PO_4 containing 10% (w/v) $CuSO_4$, and charred at 180 °C for 10 min, and lipids were quantified by photodensitometry (Shimadzu, Kyoto, Japan).

5.6. Recovery and Analysis of Covalently Bound Lipids

After sequential extraction of epidermal samples as described above, pellets were repeatedly extracted for 2 hours at 37°C with Chloroform/methanol/Water (3:7:1, v/v) followed by two additional extractions for 2 h with 95% methanol at 60 °C. The final fraction was checked for lipid content. Covalently bound lipids were released by incubation with 1 ml of 1 M KOH in 95% methanol for 2 h at 60 °C. The mixture was neutralized by adding 68.75 µl glacial acetic acid and centrifuged. The pellet was washed 2 times with 200 µl Methanol. The supernatant fractions were reunited and dried under Nitrogen stream, then diluted in Chloroform/ Methanol (1:1, v/v). Lipids were applied to prewashed (chloroform/methanol 1:1, v/v) thin-layer Silica Gel 60 plates (Merck) and developed twice using chloroform/methanol/acetic acid (190:9:1, v/v/v) as developing solvent. Quantification of lipids was performed as described above. For quantification of fatty acids, increasing amounts of palmitic acid (Fluka, Buchs, Switzerland) were applied to the plates.

5.7. Quantification of cellular DNA

Aliquots from aqueous cell homogenates (3 µl) were diluted in 97 µl TNE-buffer (10 mM Tris, 2 M NaCl, 1 mM EDTA, pH 7.4) and mixed with 100 µl bisbenzimide (fluorescence coloring Hoechst No. 33258) solution in TNE (20 µg/ml). The samples were measured in 96 well microtiter plates (Greiner Bio-One GmbH, Frickenhausen, Germany) with a *Labsystems Fluoroscan II* fluorescence photometer (Labsystems Oy, Helsinki, Finland, excitation filter 355 nm, emission filter 460 nm) and the DNA amounts were calculated relative to a standard curve.

5.8. Enzyme Assays

5.8.1. Serine palmitoyltransferase assay (van Echten-Deckert et al. 1998): Cells were harvested at the indicated time points after calcium shift in the assay buffer and cell homogenates were obtained by sonication of the cell pellet for 2 min on ice. The assay buffer contained 0.1 M Hepes (pH 7.4), 5 mM DTT, 10 mM EDTA, 50 µM pyridoxal phosphate, 1.2 mM [^{14}C]-serine (1.6 µCi), 0.15 mM palmitoyl CoA and 120 µg of cell protein in

100 µl final volume. After incubation for 10 min 37°C, reactions were terminated by addition of chloroform/methanol (5:3). The lipids were extracted by phase separation and applied to a TLC plate that was developed with chloroform/methanol, 2 M NH_3 (40:10:1). The radiolabel in 3-dehydrosphinganine was measured by scanning the TLC plate with the Phospho-imager.

5.8.2. β-Glucocerebrosidase assay (Holleran et al. 1992) (Grace et al. 1997): Harvested cells were sonicated in 0.04 M citrate/phosphate buffer (pH 5.5) containing 1 mM EDTA, 4 mM β-mercaptoethanol, 0.1 % Triton X-100, and 0.1 % sodium taurocholate, using a cup sonicator (80 watt, pulse times 30, 20 and 20 sec.). Aliquots containing equal amounts of proteins (determined suing the Bradford method) were centrifuged by 875g for 20 min. The final reaction mixture contained 4 mM 4-methylumbelliferyl-β-D-glucopyranoside (200 µl final volume) in 0.04 M citrate/phosphate buffer (pH 5.5), 1 mM EDTA, 4 mM β-mercaptoethanol, 0.25 % Triton X-100, and 0.25 % sodium taurocholate. After incubation by 37°C for 60 min, the reaction was terminated by raising the pH with the addition of 2.3 ml of a 0.1 M aqueous solution of ethylendiamine. The fluorescence of the samples was read at 448 nm. A standard solution of 4-methylumbelliferone (0-300 nM) was used for calibration of each assay. Results were expressed in nmol/min/mg protein.

5.9. RNA Extraction and Quantification

Total RNA was extracted from cultured keratinocytes and fibroblasts using the Purescript RNA isolation kit according to the supplier's instructions. The extracted RNA was suspended in sterile RNA rehydration solution and in case of contamination with DNA, RNA templates were treated with RNase free-DNase I (10 U/µl; 1 hour at 37°C and 5 min at 94°C). RNA quality was tested using a 1% standard RNA agarose gel and by measuring the optical density ratio A_{260}/A_{280} (≥ 2.0). RNA solutions were then quantified using a Genequant II unit (Amersham Pharmacia Biotech; Freiburg, Germany) and diluted to 50 ng/µl in diethyl pyrocarbonate treated sterile water and stored at –80°C.

5.10. Real-time PCR RNA Quantification

For every studied gene, a set of primers and probe (Table 1) were generated using the Primer Express 1.0 Software from Applied Biosystems. Primers were dissolved to 200 µM in diethyl pyrocarbonate treated sterile water. All fluorogenic probes contained the reporter dye FAM covalently attached to the 5'-end and the quencher dye TAMRA covalently attached to the

3'-end. Extension from 3'-end was blocked by attachment of a 3'-phosphate group. The specificity of the primers was analyzed by agarose gel electrophoresis where a single product of correct size could be observed for each primer pair. The PCR reactions were optimized for each primer-probe set before measurements.

5.11. cDNA Synthesis

The target RNA (50 ng) was reverse-transcribed using 12.5 U MuLV Reverse Transcriptase at 48°C for 30 min in the presence of 10 mM Tris-HCl (pH 8.3), 5 mM KCl, 200 µM dNTPs (each), 5 U RNasin. The reaction volume was 20 µl. Optimized concentrations of reverse primers and $MgCl_2$ were used for each specific assay. After cDNA synthesis, reverse transcriptase was denatured at 95°C for 5 min.

5.12. PCR Amplification

PCR reactions were performed with the ABI Prism 7700 sequence detection system (Applied Biosystems). 20 µl of the RT-PCR reaction from each cDNA sample were amplified in a total volume of 90 µl containing 10 mM Tris-HCl (pH 8.3), 5mM KCl, 200 µM dATP, 200 µM dCTP, 200 µM dGTP, 400 µM dUTP, 100 nM fluorogenic probe, 0.75 U AmpliTaq Gold and 0.075 U Uracil-N-glycosylase. The concentration of $MgCl_2$ (range 5 to 6.5 mM), forward and reverse primer (range 300 to 900 nM) differed for each detection of the respective mRNA due to optimization steps prior to quantification. Each amplification was performed in triplicate, using the following conditions: 2 min at 50°C and 10 min at 94°C, followed by a total of 40 two-step cycles (15 s at 94°C and 1 min at 59°C). The results were analyzed using the standard curve method as described in User Bulletin number 2 (ABI Prism 7700 sequence detection system, October 2001, Applied Biosystems). In brief, this method is based on the amplification of an endogenous control mRNA to normalize the amount of target gene mRNA in the sample and allows the correction of minor variations in input amounts of RNA samples. For all experimental samples, target amount is determined from the standard curve and divided by the amount of endogenous control. As an endogenous control we used the porphobilinogen deaminase (PBGD) gene mRNA, which was quantified in parallel in each experiment. The expression of the PBGD gene was tested in keratinocytes cultured for 8 days after calcium shift in the absence or in the presence of either SNAP or L-NAME. Its expression was found to be constant during keratinocyte differentiation (data not shown). The results were expressed as fold increase of mRNA amount of the unknown samples relative to the mRNA amount obtained for the sample at time point 0 hours. Using the PBGD

mRNA as internal control, the error bars were calculated as the standard error of the mean expressed in percent of the mean value, calculated according to User Bulletin number 2 (ABI Prism 7700 sequence detection system, October 2001, Applied Biosystems), and using 6 measurements for each sample.

5.13. Apoptosis Test

The apoptosis test was performed using the ApoAlert® caspase-3 colorimetric assay kit. The method is based on the measurement of the fluorescence in a sample after adding the specific caspase-3 substrate peptide DEVD conjugated with a chromophore pNA (DEVD-pNA). Cleavage of the substrate DEVD by caspase-3 liberated the chromophore pNA, which was then detected. The fluorescence increased proportionally to the caspase-3 activity and could be quantified using a standard curve. Cultivated keratinocytes and fibroblasts (about $2x10^6$ cells) treated either with 10 mM L-NAME or 0.2 mM SNAP were washed 3 times with cold PBS buffer and harvested. The cells were then treated as described by the manufacturer. In order to obtain a positive control of apoptosis, keratinocytes and fibroblasts were treated with actinomycin D. The results are presented in percent of the optical density at 405 nm measured for the positive control value obtained after treatment with actinomycin D. Untreated human keratinocytes and fibroblasts were used as negative controls.

6. Literature List

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Akool el, S., H. Kleinert, F. M. Hamada, M. H. Abdelwahab, U. Forstermann, J. Pfeilschifter and W. Eberhardt (2003). "Nitric oxide increases the decay of matrix metalloproteinase 9 mRNA by inhibiting the expression of mRNA-stabilizing factor HuR." *Mol Cell Biol* **23**(14): 4901-16.

Albina, J. E., S. Cui, R. B. Mateo and J. S. Reichner (1993). "Nitric oxide-mediated apoptosis in murine peritoneal macrophages." *J Immunol* **150**(11): 5080-5.

Albina, J. E., C. D. Mills, W. L. Henry, Jr. and M. D. Caldwell (1990). "Temporal expression of different pathways of 1-arginine metabolism in healing wounds." *J Immunol* **144**(10): 3877-80.

Alderton, W. K., C. E. Cooper and R. G. Knowles (2001). "Nitric oxide synthases: structure, function and inhibition." *Biochem J* **357**(Pt 3): 593-615.

Andrieu-Abadie, N., V. Gouaze, R. Salvayre and T. Levade (2001). "Ceramide in apoptosis signaling: relationship with oxidative stress." *Free Radic Biol Med* **31**(6): 717-28.

Asselineau, D., B. A. Dale and B. A. Bernard (1990). "Filaggrin production by cultured human epidermal keratinocytes and its regulation by retinoic acid." *Differentiation* **45**(3): 221-9.

Balda, M. S. and K. Matter (1998). "Tight junctions." *J Cell Sci* **111 (Pt 5)**: 541-7.

Beer, H. D., C. Munding, N. Dubois, C. Mamie, D. Hohl and S. Werner (2002). "The estrogen-responsive B box protein: a novel regulator of keratinocyte differentiation." *J Biol Chem* **277**(23): 20740-9.

Bieberich, E., T. Kawaguchi and R. K. Yu (2000). "N-acylated serinol is a novel ceramide mimic inducing apoptosis in neuroblastoma cells." *J Biol Chem* **275**(1): 177-81.

Bikle, D. D., D. Ng, C. L. Tu, Y. Oda and Z. Xie (2001). "Calcium- and vitamin D-regulated keratinocyte differentiation." *Mol Cell Endocrinol* **177**(1-2): 161-71.

Billiar, T. R. (1995). "Nitric oxide. Novel biology with clinical relevance." *Ann Surg* **221**(4): 339-49.

Bogdan, C. (2001). "Nitric oxide and the regulation of gene expression." *Trends Cell Biol* **11**(2): 66-75.

Bouwstra, J. A., P. L. Honeywell-Nguyen, G. S. Gooris and M. Ponec (2003). "Structure of the skin barrier and its modulation by vesicular formulations." *Prog Lipid Res* **42**(1): 1-36.

Brandner, J. M., S. Kief, C. Grund, M. Rendl, P. Houdek, C. Kuhn, E. Tschachler, W. W. Franke and I. Moll (2002). "Organization and formation of the tight junction system in human epidermis and cultured keratinocytes." *Eur J Cell Biol* **81**(5): 253-63.

Breiden, B. (2003). Metabolic and morpholical studies of differentiated keratinocytes. Biochemistry. Biochemistry, Rheinischen Friedrich-Wilhelms-University Bonn.

Bruch-Gerharz, D., T. Ruzicka and V. Kolb-Bachofen (1998). "Nitric oxide in human skin: current status and future prospects." *J Invest Dermatol* **110**(1): 1-7.

Byrne, C., M. Tainsky and E. Fuchs (1994). "Programming gene expression in developing epidermis." *Development* **120**(9): 2369-83.

Candi, E., S. Oddi, A. Terrinoni, A. Paradisi, M. Ranalli, A. Finazzi-Agro and G. Melino (2001). "Transglutaminase 5 cross-links loricrin, involucrin, and small proline-rich proteins in vitro." *J Biol Chem* **276**(37): 35014-23.

Davis, K. L., E. Martin, I. V. Turko and F. Murad (2001). "Novel effects of nitric oxide." *Annu Rev Pharmacol Toxicol* **41**: 203-36.

Dawn, B. and R. Bolli (2002). "Role of nitric oxide in myocardial preconditioning." *Ann N Y Acad Sci* **962**: 18-41.

6. Literature List

Dawson, V. L. and T. M. Dawson (1998). "Nitric oxide in neurodegeneration." *Prog Brain Res* **118**: 215-29.

Dimmeler, S., J. Haendeler, M. Nehls and A. M. Zeiher (1997). "Suppression of apoptosis by nitric oxide via inhibition of interleukin- 1beta-converting enzyme (ICE)-like and cysteine protease protein (CPP)- 32-like proteases." *J Exp Med* **185**(4): 601-7.

Doering, T., W. M. Holleran, A. Potratz, G. Vielhaber, P. M. Elias, K. Suzuki and K. Sandhoff (1999a). "Sphingolipid activator proteins are required for epidermal permeability barrier formation." *J Biol Chem* **274**(16): 11038-45.

Doering, T., R. L. Proia and K. Sandhoff (1999b). "Accumulation of protein-bound epidermal glucosylceramides in beta- glucocerebrosidase deficient type 2 Gaucher mice." *FEBS Lett* **447**(2-3): 167-70.

Döring, T. (1999). The Barrier of the Skin - The metabolism of ceramide and glucosylceramide in the epidermis. Department of Biochemistry. Bonn, Rheinischen Friedrich-Wilhelms-University Bonn.

Eckert, R. L., M. T. Sturniolo, A. M. Broome, M. Ruse and E. A. Rorke (2005). "Transglutaminases in epidermis." *Prog Exp Tumor Res* **38**: 115-24.

Elias, P. M., C. Cullander, T. Mauro, U. Rassner, L. Komuves, B. E. Brown and G. K. Menon (1998). "The secretory granular cell: the outermost granular cell as a specialized secretory cell." *J Investig Dermatol Symp Proc* **3**(2): 87-100.

Frank, S., B. Stallmeyer, H. Kampfer, N. Kolb and J. Pfeilschifter (1999). "Nitric oxide triggers enhanced induction of vascular endothelial growth factor expression in cultured keratinocytes (HaCaT) and during cutaneous wound repair." *Faseb J* **13**(14): 2002-14.

Fuchs, E. (1990). "Epidermal differentiation: the bare essentials." *J Cell Biol* **111**(6 Pt 2): 2807-14.

Fuchs, E. and H. Green (1981). "Regulation of terminal differentiation of cultured human keratinocytes by vitamin A." *Cell* **25**(3): 617-25.

Fukasawa, M., M. Nishijima and K. Hanada (1999). "Genetic evidence for ATP-dependent endoplasmic reticulum-to-Golgi apparatus trafficking of ceramide for sphingomyelin synthesis in Chinese hamster ovary cells." *J Cell Biol* **144**(4): 673-85.

Furukawa, F., K. Fujii, Y. Horiguchi, N. Matsuyoshi, M. Fujita, K. Toda, S. Imamura, H. Wakita, S. Shirahama and M. Takigawa (1997). "Roles of E- and P-cadherin in the human skin." *Microsc Res Tech* **38**(4): 343-52.

Furuse, M., M. Hata, K. Furuse, Y. Yoshida, A. Haratake, Y. Sugitani, T. Noda, A. Kubo and S. Tsukita (2002). "Claudin-based tight junctions are crucial for the mammalian epidermal barrier: a lesson from claudin-1-deficient mice." *J Cell Biol* **156**(6): 1099-111.

Geilen, C. C., S. Barz and M. Bektas (2001). "Sphingolipid signaling in epidermal homeostasis. Current knowledge and new therapeutic approaches in dermatology." *Skin Pharmacol Appl Skin Physiol* **14**(5): 261-71.

Gibbs, S., J. Vicanova, J. Bouwstra, D. Valstar, J. Kempenaar and M. Ponec (1997). "Culture of reconstructed epidermis in a defined medium at 33 degrees C shows a delayed epidermal maturation, prolonged lifespan and improved stratum corneum." *Arch Dermatol Res* **289**(10): 585-95.

Gow, A. J. and H. Ischiropoulos (2001). "Nitric oxide chemistry and cellular signaling." *J Cell Physiol* **187**(3): 277-82.

Grace, E. M., R. J. Desnick and G. M. Pastores (1997). "Identification and expression of acid b-glucosidase mutations causing severe type 1 and neurologic type 2 gaucher disease in non-jewish patients." *Am Soc Clin Inv* **99**(10): 2530-37.

6. Literature List

Hammerberg, C., Z. Bata-Csorgo, J. J. Voorhees and K. D. Cooper (1998). "IL-1 and IL-1 receptor antagonist regulation during keratinocyte cell cycle and differentiation in normal and psoriatic epidermis." *Arch Dermatol Res* **290**(7): 367-74.

Hanada, K., K. Kumagai, S. Yasuda, Y. Miura, M. Kawano, M. Fukasawa and M. Nishijima (2003). "Molecular machinery for non-vesicular trafficking of ceramide." *Nature* **426**(6968): 803-9.

Hanley, K., Y. Jiang, W. M. Holleran, P. M. Elias, M. L. Williams and K. R. Feingold (1997). "Glucosylceramide metabolism is regulated during normal and hormonally stimulated epidermal barrier development in the rat." *J Lipid Res* **38**(3): 576-84.

Hannun, Y. A. (1996). "Functions of ceramide in coordinating cellular responses to stress." *Science* **274**(5294): 1855-9.

Hannun, Y. A., C. Luberto and K. M. Argraves (2001). "Enzymes of sphingolipid metabolism: from modular to integrative signaling." *Biochemistry* **40**(16): 4893-903.

Haratake, A., Y. Uchida, K. Mimura, P. M. Elias and W. M. Holleran (1997). "Intrinsically aged epidermis displays diminished UVB-induced alterations in barrier function associated with decreased proliferation." *J Invest Dermatol* **108**(3): 319-23.

Hassler, D. F. and R. M. Bell (1993). "Ceramidases: enzymology and metabolic roles." *Adv Lipid Res* **26**: 49-57.

Hendrich, M. P., A. K. Upadhyay, J. Riga, D. M. Arciero and A. B. Hooper (2002). "Spectroscopic characterization of the NO adduct of hydroxylamine oxidoreductase." *Biochemistry* **41**(14): 4603-11.

Hendrix, S. W., J. V. Rogers and B. E. Hull (1998). "Differential response of basal keratinocytes in a human skin equivalent to ultraviolet irradiation." *Arch Dermatol Res* **290**(8): 420-4.

Hennings, H., D. Michael, C. Cheng, P. Steinert, K. Holbrook and S. H. Yuspa (1980). "Calcium regulation of growth and differentiation of mouse epidermal cells in culture." *Cell* **19**(1): 245-54.

Holleran, W. M., Y. Takagi, G. Imokawa, S. Jackson, J. M. Lee and P. M. Elias (1992). "beta-Glucocerebrosidase activity in murine epidermis: characterization and localization in relation to differentiation." *J Lipid Res* **33**(8): 1201-9.

Holleran, W. M., Y. Takagi, G. K. Menon, S. M. Jackson, J. M. Lee, K. R. Feingold and P. M. Elias (1994). "Permeability barrier requirements regulate epidermal beta-glucocerebrosidase." *J Lipid Res* **35**(5): 905-12.

Holleran, W. M., Y. Takagi, G. K. Menon, G. Legler, K. R. Feingold and P. M. Elias (1993). "Processing of epidermal glucosylceramides is required for optimal mammalian cutaneous permeability barrier function." *J Clin Invest* **91**(4): 1656-64.

Huang, P. L. (2000). "Mouse models of nitric oxide synthase deficiency." *J Am Soc Nephrol* **11 Suppl 16**: S120-3.

Huang, Z., P. L. Huang, N. Panahian, T. Dalkara, M. C. Fishman and M. A. Moskowitz (1994). "Effects of cerebral ischemia in mice deficient in neuronal nitric oxide synthase." *Science* **265**(5180): 1883-5.

Huelsken, J., R. Vogel, B. Erdmann, G. Cotsarelis and W. Birchmeier (2001). "beta-Catenin controls hair follicle morphogenesis and stem cell differentiation in the skin." *Cell* **105**(4): 533-45.

Huwiler, A., J. Pfeilschifter and H. van den Bosch (1999). "Nitric oxide donors induce stress signaling via ceramide formation in rat renal mesangial cells." *J Biol Chem* **274**(11): 7190-5.

Ignarro, L. J., G. Cirino, A. Casini and C. Napoli (1999). "Nitric oxide as a signaling molecule in the vascular system: an overview." *J Cardiovasc Pharmacol* **34**(6): 879-86.

6. Literature List

Jeckel, D., A. Karrenbauer, K. N. Burger, G. van Meer and F. Wieland (1992). "Glucosylceramide is synthesized at the cytosolic surface of various Golgi subfractions." *J Cell Biol* **117**(2): 259-67.

Jennemann, R., R. Sandhoff, L. Langbein, S. Kaden, U. Rothermel, H. Gallala, K. Sandhoff, H. Wiegandt and H. J. Gröne (2006). "Integrity and barrier function of the skin depend on glucosylceramide synthesis in epidermis." **in preparation**.

Jennemann, R., R. Sandhoff, S. Wang, E. Kiss, N. Gretz, C. Zuliani, A. Martin-Villalba, R. Jager, H. Schorle, M. Kenzelmann, M. Bonrouhi, H. Wiegandt and H. J. Grone (2005). "Cell-specific deletion of glucosylceramide synthase in brain leads to severe neural defects after birth." *Proc Natl Acad Sci U S A* **102**(35): 12459-64.

Kalinin, A. E., A. V. Kajava and P. M. Steinert (2002). "Epithelial barrier function: assembly and structural features of the cornified cell envelope." *Bioessays* **24**(9): 789-800.

Kim, P. K., Y. G. Kwon, H. T. Chung and Y. M. Kim (2002). "Regulation of caspases by nitric oxide." *Ann N Y Acad Sci* **962**: 42-52.

Kirfel, J., T. M. Magin and J. Reichelt (2003). "Keratins: a structural scaffold with emerging functions." *Cell Mol Life Sci* **60**(1): 56-71.

Krischel, V., D. Bruch-Gerharz, C. Suschek, K. D. Kroncke, T. Ruzicka and V. Kolb-Bachofen (1998). "Biphasic effect of exogenous nitric oxide on proliferation and differentiation in skin derived keratinocytes but not fibroblasts." *J Invest Dermatol* **111**(2): 286-91.

Kumagai, K., S. Yasuda, K. Okemoto, M. Nishijima, S. Kobayashi and K. Hanada (2005). "CERT mediates intermembrane transfer of various molecular species of ceramides." *J Biol Chem* **280**(8): 6488-95.

Kuo, M. L., Y. P. Chau, J. H. Wang and S. G. Shiah (1996). "Inhibitors of poly(ADP-ribose) polymerase block nitric oxide-induced apoptosis but not differentiation in human leukemia HL-60 cells." *Biochem Biophys Res Commun* **219**(2): 502-8.

Lala, P. K. and C. Chakraborty (2001). "Role of nitric oxide in carcinogenesis and tumour progression." *Lancet Oncol* **2**(3): 149-56.

Langmann, T., C. Buechler, S. Ries, A. Schaeffler, C. Aslanidis, M. Schuierer, M. Weiler, K. Sandhoff, P. J. de Jong and G. Schmitz (1999). "Transcription factors Sp1 and AP-2 mediate induction of acid sphingomyelinase during monocytic differentiation." *J Lipid Res* **40**(5): 870-80.

Lannert, H., C. Bunning, D. Jeckel and F. T. Wieland (1994). "Lactosylceramide is synthesized in the lumen of the Golgi apparatus." *FEBS Lett* **342**(1): 91-6.

Lannert, H., K. Gorgas, I. Meissner, F. T. Wieland and D. Jeckel (1998). "Functional organization of the Golgi apparatus in glycosphingolipid biosynthesis. Lactosylceramide and subsequent glycosphingolipids are formed in the lumen of the late Golgi." *J Biol Chem* **273**(5): 2939-46.

Lee, H., E. J. Goetzl and S. An (2000). "Lysophosphatidic acid and sphingosine 1-phosphate stimulate endothelial cell wound healing." *Am J Physiol Cell Physiol* **278**(3): C612-8.

Lee, R. H., D. Efron, U. Tantry and A. Barbul (2001). "Nitric oxide in the healing wound: a time-course study." *J Surg Res* **101**(1): 104-8.

Li, E. R., D. M. Owens, P. Djian and F. M. Watt (2000). "Expression of involucrin in normal, hyperproliferative and neoplastic mouse keratinocytes." *Exp Dermatol* **9**(6): 431-8.

Li, J., C. A. Bombeck, S. Yang, Y. M. Kim and T. R. Billiar (1999). "Nitric oxide suppresses apoptosis via interrupting caspase activation and mitochondrial dysfunction in cultured hepatocytes." *J Biol Chem* **274**(24): 17325-33.

Liew, F. Y. and F. E. Cox (1991). "Nonspecific defence mechanism: the role of nitric oxide." *Immunol Today* **12**(3): A17-21.

6. Literature List

Limat, A., D. Mauri and T. Hunziker (1996). "Successful treatment of chronic leg ulcers with epidermal equivalents generated from cultured autologous outer root sheath cells." *J Invest Dermatol* **107**(1): 128-35.

Lui, K., C. Commens, R. Choong and R. Jaworski (1988). "Collodion babies with Gaucher's disease." *Arch Dis Child* **63**(7): 854-6.

Macheleidt, O., H. W. Kaiser and K. Sandhoff (2002). "Deficiency of epidermal protein-bound omega-hydroxyceramides in atopic dermatitis." *J Invest Dermatol* **119**(1): 166-73.

Maeda, I., T. Takano, F. Matsuzuka, T. Maruyama, T. Higashiyama, G. Liu, K. Kuma and N. Amino (1999). "Rapid screening of specific changes in mRNA in thyroid carcinomas by sequence specific-differential display: decreased expression of acid ceramidase mRNA in malignant and benign thyroid tumors." *Int J Cancer* **81**(5): 700-4.

Manggau, M., D. S. Kim, L. Ruwisch, R. Vogler, H. C. Korting, M. Schafer-Korting and B. Kleuser (2001). "1Alpha,25-dihydroxyvitamin D3 protects human keratinocytes from apoptosis by the formation of sphingosine-1-phosphate." *J Invest Dermatol* **117**(5): 1241-9.

Matthews, J. R., C. H. Botting, M. Panico, H. R. Morris and R. T. Hay (1996). "Inhibition of NF-kappaB DNA binding by nitric oxide." *Nucleic Acids Res* **24**(12): 2236-42.

McCabe, T. J., D. Fulton, L. J. Roman and W. C. Sessa (2000). "Enhanced electron flux and reduced calmodulin dissociation may explain "calcium-independent" eNOS activation by phosphorylation." *J Biol Chem* **275**(9): 6123-8.

Merrill, A. H., Jr. and E. Wang (1986). "Biosynthesis of long-chain (sphingoid) bases from serine by LM cells. Evidence for introduction of the 4-trans-double bond after de novo biosynthesis of N-acylsphinganine(s)." *J Biol Chem* **261**(8): 3764-9.

Messmer, U. K. and B. Brune (1996). "Nitric oxide (NO) in apoptotic versus necrotic RAW 264.7 macrophage cell death: the role of NO-donor exposure, NAD+ content, and p53 accumulation." *Arch Biochem Biophys* **327**(1): 1-10.

Michel, C. and G. van Echten-Deckert (1997). "Conversion of dihydroceramide to ceramide occurs at the cytosolic face of the endoplasmic reticulum." *FEBS Lett* **416**(2): 153-5.

Michel, C., G. van Echten-Deckert, J. Rother, K. Sandhoff, E. Wang and A. H. Merrill, Jr. (1997). "Characterization of ceramide synthesis. A dihydroceramide desaturase introduces the 4,5-trans-double bond of sphingosine at the level of dihydroceramide." *J Biol Chem* **272**(36): 22432-7.

Moncada, S. and J. D. Erusalimsky (2002). "Does nitric oxide modulate mitochondrial energy generation and apoptosis?" *Nat Rev Mol Cell Biol* **3**(3): 214-20.

Moncada, S. and A. Higgs (1993). "The L-arginine-nitric oxide pathway." *N Engl J Med* **329**(27): 2002-12.

Montagna, W., A. M. Kligman and K. S. Carlisle (1992). Atlas of normal human skin, Springer Verlag, Berlin-Heidelberg-New York.

Morita, K., M. Itoh, M. Saitou, Y. Ando-Akatsuka, M. Furuse, K. Yoneda, S. Imamura, K. Fujimoto and S. Tsukita (1998). "Subcellular distribution of tight junction-associated proteins (occludin, ZO-1, ZO-2) in rodent skin." *J Invest Dermatol* **110**(6): 862-6.

Morita, K. and Y. Miyachi (2003). "Tight junctions in the skin." *J Dermatol Sci* **31**(2): 81-9.

Motta, S., M. Monti, S. Sesana, R. Caputo, S. Carelli and R. Ghidoni (1993). "Ceramide composition of the psoriatic scale." *Biochim Biophys Acta* **1182**(2): 147-51.

Nathan, C. and Q. W. Xie (1994). "Regulation of biosynthesis of nitric oxide." *J Biol Chem* **269**(19): 13725-8.

Nemes, Z. and P. M. Steinert (1999). "Bricks and mortar of the epidermal barrier." *Exp Mol Med* **31**(1): 5-19.

6. Literature List

Perry, D. K. (2000). "The role of de novo ceramide synthesis in chemotherapy-induced apoptosis." *Ann N Y Acad Sci* **905**: 91-6.

Ponec, M., A. Weerheim, J. Kempenaar, A. Mulder, G. S. Gooris, J. Bouwstra and A. M. Mommaas (1997). "The formation of competent barrier lipids in reconstructed human epidermis requires the presence of vitamin C." *J Invest Dermatol* **109**(3): 348-55.

Ponec, M., A. Weerheim, P. Lankhorst and P. Wertz (2003). "New acylceramide in native and reconstructed epidermis." *J Invest Dermatol* **120**(4): 581-8.

Pummi, K., M. Malminen, H. Aho, S. L. Karvonen, J. Peltonen and S. Peltonen (2001). "Epidermal tight junctions: ZO-1 and occludin are expressed in mature, developing, and affected skin and in vitro differentiating keratinocytes." *J Invest Dermatol* **117**(5): 1050-8.

Rheinwald, J. G. and H. Green (1975). "Serial cultivation of strains of human epidermal keratinocytes: the formation of keratinizing colonies from single cells." *Cell* **6**(3): 331-43.

Riezman, H. and G. van Meer (2004). "Lipid pickup and delivery." *Nat Cell Biol* **6**(1): 15-6.

Roop, D. R., H. Huitfeldt, A. Kilkenny and S. H. Yuspa (1987). "Regulated expression of differentiation-associated keratins in cultured epidermal cells detected by monospecific antibodies to unique peptides of mouse epidermal keratins." *Differentiation* **35**(2): 143-50.

Rossi, A., M. V. Catani, E. Candi, F. Bernassola, P. Puddu and G. Melino (2000). "Nitric oxide inhibits cornified envelope formation in human keratinocytes by inactivating transglutaminases and activating protein 1." *J Invest Dermatol* **115**(4): 731-9.

Rossi, A., S. I. Jang, R. Ceci, P. M. Steinert and N. G. Markova (1998). "Effect of AP1 transcription factors on the regulation of transcription in normal human epidermal keratinocytes." *J Invest Dermatol* **110**(1): 34-40.

Rother, J., G. van Echten, G. Schwarzmann and K. Sandhoff (1992). "Biosynthesis of sphingolipids: dihydroceramide and not sphinganine is desaturated by cultured cells." *Biochem Biophys Res Commun* **189**(1): 14-20.

Rudolf, M., O. Einsle, F. Neese and P. M. Kroneck (2002). "Pentahaem cytochrome c nitrite reductase: reaction with hydroxylamine, a potential reaction intermediate and substrate." *Biochem Soc Trans* **30**(4): 649-53.

Sandau, K. and B. Brune (1996). "The dual role of S-nitrosoglutathione (GSNO) during thymocyte apoptosis." *Cell Signal* **8**(3): 173-7.

Savini, I., M. V. Catani, A. Rossi, G. Duranti, G. Melino and L. Avigliano (2002). "Characterization of keratinocyte differentiation induced by ascorbic acid: protein kinase C involvement and vitamin C homeostasis." *J Invest Dermatol* **118**(2): 372-9.

Schmuth, M., M. Q. Man, F. Weber, W. Gao, K. R. Feingold, P. Fritsch, P. M. Elias and W. M. Holleran (2000). "Permeability barrier disorder in Niemann-Pick disease: sphingomyelin- ceramide processing required for normal barrier homeostasis." *J Invest Dermatol* **115**(3): 459-66.

Schuette, C. G., T. Doering, T. Kolter and K. Sandhoff (1999). "The glycosphingolipidoses-from disease to basic principles of metabolism." *Biol Chem* **380**(7-8): 759-66.

Shimizu, Y., M. Sakai, Y. Umemura and H. Ueda (1997). "Immunohistochemical localization of nitric oxide synthase in normal human skin: expression of endothelial-type and inducible-type nitric oxide synthase in keratinocytes." *J Dermatol* **24**(2): 80-7.

Siragy, H. M., R. A. Johns, M. J. Peach and R. M. Carey (1992). "Nitric oxide alters renal function and guanosine 3',5'-cyclic monophosphate." *Hypertension* **19**(6 Pt 2): 775-9.

Stallmeyer, B., H. Kampfer, N. Kolb, J. Pfeilschifter and S. Frank (1999). "The function of nitric oxide in wound repair: inhibition of inducible nitric oxide-synthase severely impairs wound reepithelialization." *J Invest Dermatol* **113**(6): 1090-8.

6. Literature List

Steinert, P. M., J. S. Cantieri, D. C. Teller, J. D. Lonsdale-Eccles and B. A. Dale (1981). "Characterization of a class of cationic proteins that specifically interact with intermediate filaments." *Proc Natl Acad Sci U S A* **78**(7): 4097-101.

Stewart, M. E. and D. T. Downing (1999). "A new 6-hydroxy-4-sphingenine-containing ceramide in human skin." *J Lipid Res* **40**(8): 1434-9.

Stoffel, W., D. LeKim and G. Sticht (1968). "Metabolism of sphingosine bases, V. Biosynthesis of dihydrosphingosine in vitro." *Hoppe Seylers Z Physiol Chem* **349**(8): 664-70.

Sun, T. T. and H. Green (1976). "Differentiation of the epidermal keratinocyte in cell culture: formation of the cornified envelope." *Cell* **9**(4 Pt 1): 511-21.

Suschek, C. V., V. Krischel, D. Bruch-Gerharz, D. Berendji, J. Krutmann, K. D. Kroncke and V. Kolb-Bachofen (1999). "Nitric oxide fully protects against UVA-induced apoptosis in tight correlation with Bcl-2 up-regulation." *J Biol Chem* **274**(10): 6130-7.

Tabuchi, A., K. Sano, E. Oh, T. Tsuchiya and M. Tsuda (1994). "Modulation of AP-1 activity by nitric oxide (NO) in vitro: NO-mediated modulation of AP-1." *FEBS Lett* **351**(1): 123-7.

Teumer, K., K. Zezulak and H. Green (1994).

Uchida, Y., M. Behne, D. Quiec, P. M. Elias and W. M. Holleran (2001). "Vitamin C stimulates sphingolipid production and markers of barrier formation in submerged human keratinocyte cultures." *J Invest Dermatol* **117**(5): 1307-13.

Uchida, Y., M. Hara, H. Nishio, E. Sidransky, S. Inoue, F. Otsuka, A. Suzuki, P. M. Elias, W. M. Holleran and S. Hamanaka (2000). "Epidermal sphingomyelins are precursors for selected stratum corneum ceramides." *J Lipid Res* **41**(12): 2071-82.

Uchida, Y., A. D. Nardo, V. Collins, P. M. Elias and W. M. Holleran (2003). "De novo ceramide synthesis participates in the ultraviolet B irradiation-induced apoptosis in undifferentiated cultured human keratinocytes." *J Invest Dermatol* **120**(4): 662-9.

van Echten-Deckert, G., A. Giannis, A. Schwarz, A. H. Futerman and K. Sandhoff (1998). "1-Methylthiodihydroceramide, a novel analog of dihydroceramide, stimulates sphinganine degradation resulting in decreased de novo sphingolipid biosynthesis." *J Biol Chem* **273**(2): 1184-91.

Vann, L. R., S. Twitty, S. Spiegel and S. Milstien (2000). "Divergence in regulation of nitric-oxide synthase and its cofactor tetrahydrobiopterin by tumor necrosis factor-alpha. Ceramide potentiates nitric oxide synthesis without affecting GTP cyclohydrolase I activity." *J Biol Chem* **275**(18): 13275-81.

Waldman, S. A. and F. Murad (1988). "Biochemical mechanisms underlying vascular smooth muscle relaxation: the guanylate cyclase-cyclic GMP system." *J Cardiovasc Pharmacol* **12 Suppl 5**: S115-8.

Watanabe, R., K. Wu, P. Paul, D. L. Marks, T. Kobayashi, M. R. Pittelkow and R. E. Pagano (1998). "Up-regulation of glucosylceramide synthase expression and activity during human keratinocyte differentiation." *J Biol Chem* **273**(16): 9651-5.

Watt, F. M. (1988). "The epidermal keratinocyte." *Bioessays* **8**(5): 163-7.

Watt, F. M. (1989). "Terminal differentiation of epidermal keratinocytes." *Curr Opin Cell Biol* **1**(6): 1107-15.

Weerheim, A. and M. Ponec (2001). "Determination of stratum corneum lipid profile by tape stripping in combination with high-performance thin-layer chromatography." *Arch Dermatol Res* **293**(4): 191-9.

Wertz, P. W. and D. T. Downing (1987). "Covalently bound omega-hydroxyacylsphingosine in the stratum corneum." *Biochim Biophys Acta* **917**(1): 108-11.

Wertz, P. W., K. C. Madison and D. T. Downing (1989). "Covalently bound lipids of human stratum corneum." *J Invest Dermatol* **92**(1): 109-11.

6. Literature List

Wertz, P. W., M. C. Miethke, S. A. Long, J. S. Strauss and D. T. Downing (1985). "The composition of the ceramides from human stratum corneum and from comedones." *J Invest Dermatol* **84**(5): 410-2.

Yamashita, T., R. Wada, T. Sasaki, C. Deng, U. Bierfreund, K. Sandhoff and R. L. Proia (1999). "A vital role for glycosphingolipid synthesis during development and differentiation." *Proc Natl Acad Sci U S A* **96**(16): 9142-7.

Zamora, R., Y. Vodovotz and T. R. Billiar (2000). "Inducible nitric oxide synthase and inflammatory diseases." *Mol Med* **6**(5): 347-73.

Zhou, H., S. A. Summers, M. J. Birnbaum and R. N. Pittman (1998). "Inhibition of Akt kinase by cell-permeable ceramide and its implications for ceramide-induced apoptosis." *J Biol Chem* **273**(26): 16568-75.